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ENDOCRINOLOGY

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HORMONE SECRETION BY THE IMMATURE MOUSE OVARY AFTER GONADOTROPHIC STIMULATION

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Although the secretion of progesterone is usually associated with the presence of luteal tissue in the ovary, there is a growing body of evidence that the ovary can produce this hormone prior to ovulation and formation of a corpus luteum. Dempsey, Hertz and Young (1936) were able to induce more nearly normal sexual receptivity in ovariectomized guinea pigs by using estrogen and progesterone than by using estrogen alone. This observation has been extended to the rat (Boling and Blandau, 1939) and the mouse (Ring, 1944). The possibility that progesterone is secreted prior to ovulation and that it synergizes with estrogen to produce sexual receptivity was suggested. Astwood (1939) investigated the water content of the rat uterus during the estrous cycle. He found that it increased late in diestrus, decreased sharply at about the time of proestrus, and was at a low level at ovulation. He attributed the rise in water content to estrogen, and the decline to an antagonism of estrogen by progesterone.

More direct evidence of a preovulatory secretion was obtained by assaying follicular fluid for progestational activity. Duyvené de Wit (1942) assayed follicular fluid from sows, cows and humans for progestational activity using the bitterling ovipositor test and found them all positive. Hooker and Forbes (1947) found progestational activity in the follicular fluid of the sow ovarian follicle using their mouse intrauterine assay technique. Van Dyke and Gustavson (1929) made extracts of follicular fluid from swine ovaries and injected them into female cats. A photomicrograph of a section of the uterus of one of the injected cats shows an intense glandular response of the type elicited by progesterone. Unfortunately, none of these authors furnished data as to the size or morphology of the follicles from which the fluid was collected.

The present study was made to obtain more precise information with respect to the sequence of secretion of estrogen and progesterone after the administration of a gonadotrophin. The mouse was used as the experi-

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mental animal because the morphological responses of the endometrium provide reliable and specific criteria for the presence of estrogen and progesterone (Hooker, 1945). Under the influence of progesterone the stromal nuclei of the endometrium become vesicular, the chromatin becomes less dense, and a nucleolus appears. In the presence of estrogen the cells of the endometrial epithelium heighten and begin to proliferate, causing the epithelium to become pseudostratified; mitotic figures are found in the glandular epithelium; and the stroma becomes edematous.

MATERIALS AND METHOD

The mice used were members of the Strong A strain, and were 21 days old at the time of the administration of the gonadotrophin. The gonadotrophin used was an extremely potent preparation of equine serum origin,[1] and was given as a single subcutaneous injection; the dose contained in 0.25 ml. of distilled water. At autopsy the ovaries and uteri were dissected free of fat under a dissecting microscope, weighed on a torsion balance, and fixed in Bouin's solution. One ovary from each mouse was examined in serial sections and one uterine cornu of each mouse was examined in sections taken from the midportion. The presence of estrogen and progesterone was assessed by the morphological criteria listed above.

RESULTS

The first objective was determination of the ovarian and uterine weight responses to the gonadotrophin at many dose levels, and to determine if a functional correlation of the ovarian and uterine morphology could be made. Twenty-three levels were tested; these ranged from 0.34 mg. to 0.00007 mg. All mice were autopsied 72 hours after the injection.

The ovarian and uterine weights are summarized for 14 of these levels in Table 1. At all levels above 1.1 μg. the gonadotrophin produced corpus luteum formation and an increase in ovarian weight. At the 1.1 μg. level, and at all levels below that, there was no increase in ovarian weight, nor was there microscopic evidence of luteinization of the granulosa or theca interna. The increase in uterine weight at all the subluteinizing levels indicated that ovarian hormones were being secreted. The endometria at all dose levels showed morphological responses qualitatively indicative of both estrogenic and progestational activity. No attempt was made to evaluate these quantitatively.

A second group of 28 mice were given 1.1 μg of mare serum gonadotrophin, previously found to be subluteinizing. These animals were killed 12, 24, 36, 48, 60, 72 and 120 hours after the injection.

As shown in Table 2, there was no significant increase in ovarian weight of the experimental mice over that of the controls. Although no quantitative study was made, intact antrum-containing follicles appeared to be

[1] The equine serum gonadotrophin used was lot AH-300 of Gonadogen generously supplied by Dr. William J. Haines of the Upjohn Company, Kalamazoo, Michigan. This preparation was said to assay 51 C.N.U. per milligram.

TABLE 1. OVARIAN AND UTERINE WEIGHT RESPONSES OF 21-DAY OLD A STRAIN MICE 72 HOURS AFTER PMS ADMINISTRATION

Number of mice	Mg. PMS injected	Av. ovarian wt. (mg.)	Av. uterine wt. (mg.)
5	0.34	18.2	40.5
5	0.27	13.2	35.3
5	0.13	13.2	39.1
5	0.067	17.8	43.2
4	0.033	9.8	40.9
5	0.0165	11.9	39.9
5	0.0088	8.5	39.9
4	0.0044	7.3	31.2
5	0.0022	6.9	39.1
6	0.0011	4.5*	47.3
5	0.0005	5.2*	41.7
5	0.00033	5.0*	42.9
4	0.00017	4.4*	21.6
6	0.00007	4.4*	12.2
15	None	4.5*	7.7

* No luteinization.

somewhat more numerous in the mice killed 12 hours post-injection than in the controls and the other experimental groups. The ova of atretic follicles of the injected mice frequently showed maturation changes such as spindle formation and polar bodies. No differences between the theca interna and the granulosa of the injected animals and the control animals were seen.

In Table 2 the morphological responses of the endometrium to estrogen and progestin are arbitrarily rated as to their intensity. At 12 hours post-injection the subepithelial stromal nuclei had undergone definite progestational change. There was the barest indication of stromal edema, the epithelium was barely heightened, and there were no mitoses in the luminal or glandular epithelium. In view of the temporal and quantitative data of Hooker (1945) and Hooker and Forbes (1947) it seems reasonable to assume that the response of the stromal nuclei here were indicative of a substantial amount of progestin having been present for several hours. The trace of stromal edema and lack of epithelial proliferation would indicate

TABLE 2. OVARIAN AND UTERINE RESPONSES OF 21-DAY OLD A STRAIN MICE TO 1.1 μG. OF PMS

Number of mice	Hours post-inj.	Av. ovarian wt. (mg.)	Av. uterine wt. (mg.)	Endometrial response to	
				Estrogen	Progesterone
4	12	4.0	11.9	trace	+
4	24	5.1	20.8	+	++
4	36	4.6	30.6	++	++
6	48	4.9	57.1	+++	++
4	60	3.8	68.6	++++	++
4	72	5.1	54.8	+++	++
2	120	4.9	29.9	+	+++
15	controls	4.5	7.7	—	—

that the level of estrogen secretion was extremely low or been underway only a short time.

At 24 hours post-injection the progestational response in the stromal nuclei had become general throughout the endometrium. The morphological responses of the endometrium to estrogen were definite. There was stromal edema, and mitotic divisions were numerous in both the luminal and glandular epithelium.

At 36 hours post-injection the stromal edema had become intense, and the epithelium had become pseudostratified. The progestational response remained fully developed. From 48 to 60 hours the only change was the increasing edema. The only interpretation that can be made is qualitative. The uterine morphology indicated that progesterone and estrogen were acting during this period. At 72 hours the uterine weight declined, the edema lessened, and polymorphonuclear eosinophils were present in the stroma. These findings suggest that the ovarian secretion in response to the PMS is at an end.

The uterus of a mouse killed after 120 hours had reverted to the castrate-like condition seen in the controls, that is the epithelium was columnar and the stromal nuclei were small, fusiform and possessed dense chromatin.

Finally, to determine whether the adrenal was the source of the steroids responsible for the uterine morphological responses, five 21-day old mice were ovariectomized and were given single subcutaneous injections of 2.0 micrograms of the mare serum preparation used above. At the same time 3 intact mice of the same age were given a similar injection. All mice were autopsied 72 hours after the injection. The average uterine weight of the ovariectomized, PMS-injected mice was 6.2 mg. which was in the range of the uninjected controls used above. The uteri of the PMS treated ovariectomized mice showed none of the features characteristically elicited by estrogen and progesterone, while all of the intact mice showed a positive progesterone and estrogen response.

DISCUSSION

At the outset of this work it was expected that when the dose of the hormone was reduced below a non-luteinizing level, there would be no secretion of progestin; and, as a consequence, the endometria of mice given such doses would show only the typical estrogen changes and no stromal nuclei differentiation. Such findings would be expected if the current "one-two" hypothesis of ovarian secretion, which postulates the ovary secretes first estrogen and then progestin, is valid. Since progestin secretion was indicated even at the minimal effective dose, the second phase of this experiment was designed to test this hypothesis temporally after a gonadotrophic stimulus. The endometrial responses of the mice killed at intervals after the injection of the gonadotrophin indicated that progestin began to circulate as early as estrogen. This would seem to be best interpreted by

assuming that the cells acted upon by the gonadotrophin produce estrogen and progestin simultaneously.

Zondek and Sulman (1945) have reviewed their work on the interval between the administration of gonadotrophic hormone and appearance of the reaction. They concluded that although ovarian hyperemia in the rat may appear as early as two hours after the administration of chorionic gonadotrophin, estrogen is not released into the blood stream until 27 hours after the initiation of treatment, and progesterone even later. Their data on estrogen release is based on the time of vaginal cornification. The rapidity of the appearance of the uterine morphological manifestations of ovarian secretion indicated that the ovary of the immature mouse can release hormones less than 12 hours after stimulation. When one considers that the ovarian hormones must be transported to the uterus, and that a period of time must elapse before cellular differentiation occurs in response to the hormones, it is reasonable to assume that ovarian secretion begins shortly after the initiation of the trophic stimulus. In addition, this experiment demonstrates the uterus responds more rapidly than the vagina to ovarian hormones, vaginal opening occurred in these mice 36 to 48 hours after the injection.

Most of the work quoted in the introduction suggests that progestin is secreted in the interval just prior to ovulation. Since the cells of the theca interna of the preovulatory follicles of most mammals undergo differentiation and morphologically resemble luteal cells (Corner, 1946), that evidence was not contrary to the accepted view that progestin secretion is a function of the luteal cell. However, Forbes (1950) and Bryans (1951) have reported finding circulating progesterone in the cycling human and monkey during the early part of the follicular phase which is prior to any known thecal differentiation. If one assumes that the criteria used here for assessing the presence of circulating progesterone are valid, the observations indicate that the presence of a definite luteal cell is not necessary for the secretion of progestin.

The mice used here secreted ovarian hormones in the absence of any microscopically detectable ovarian differentiation. What was the site of hormone synthesis? There was no glandular appearing interstitial tissue in mice of this age, and there was no thecal differentiation. Therefore, the possibility of the granulosa cells being the site of synthesis perhaps should not be disregarded. Since the ultimate fate of the granulosa cell is either atretic degeneration or luteinization, it does not seem unreasonable to assume that given a suitable stimulus, it can secrete progestin prior to its differentiation into a definitive luteal cell. Thus to consider the follicle and corpus luteum as separate and distinct physiological entities may not be sound.

It should be emphasized that in the strain of mice used here the theca interna did not differentiate after gonadotrophic stimulation in the manner

usually seen in other mammals. This has been true with gonadotrophins of sheep, swine and horse anterior pituitary that were given to 21-day old mice of this strain (Green, unpublished work). In this respect the mouse differs from its close relative the rat.

Although Cartland and Nelson (1938) reported that a mare serum gonadotrophic preparation extracted in a manner similar to the preparation used here possessed no significant ACTH activity, the possibility of adrenal participation was investigated and found lacking.

SUMMARY

Pregnant mare serum gonadotrophin was given in single subcutaneous injections to mice 21 days old. The dose level ranged from 340 micrograms (17.7 C.N.U.) down to 0.07 micrograms (0.004 C.N.U.). Above a level of 1.1 micrograms it caused corpora lutea formation; from that level and down to the minimal effective dose the ovary did not respond by weight increase or morphological change. However, the ovary did respond physiologically at the subluteinizing levels as evidenced by marked uterine weight increase and morphological changes. At all dose levels, including the subluteinizing levels, there were changes in the endometrium indicative of the presence of both progestin and estrogen.

Another series of mice were given single subcutaneous injections of a subluteinizing dose of the same gonadotrophic preparation (1.1 micrograms or 0.06 C.N.U.) and sacrificed 12, 24, 36, 48, 60, 72, and 120 hours later. There was no distinct change in the ovarian morphology. However, there was an increase in the uterine weight. Endometrial morphology indicated that progestin was present throughout the experiment. Only a slight indication of an estrogen response was present at 12 hours post-injection; the response increased until 60 hours and then declined. It appears that estrogen and progestin were secreted simultaneously in response to the gonadotrophin.

A luteinizing dose of this preparation had no effect on the uterine morphology of ovariectomized mice, ruling out the possibility of adrenal participation.

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EXPERIMENTAL STUDIES ON THE RELATIONS BETWEEN ADRENAL CORTEX, PLASMA LIPIDS, AND ATHEROSCLEROSIS[1]

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OVER 100 years ago Virchow described the development of endarteritis deformans, a term used by him for atherosclerosis, and observed the alteration of the tissue ground substance in the evolution of this condition. Research in this field has been greatly influenced by the observation that feeding cholesterol resulted in the production of severe atherosclerosis in the rabbit (Anitschkow, 1913), as well as in other animal species (Davidson, 1951). Recently there has been a revival of interest in the role of the ground substance in atherogenesis (Klemperer, 1953).

Our previous work showed that prolonged administration of large doses of corticotropin or cortisone produced hypercholesteremia, elevation of other serum lipid fractions and lactescence of serum in man (Adlersberg *et al.*, 1950; 1951). These findings have been confirmed in the rabbit (Kobernick and More, 1950; Adlersberg *et al.*, 1951; Rich, 1951). In studies on the effect of corticotropin, cortisone and hydrocortisone on plasma lipids of the cholesterol fed rabbit marked enhancements of hypercholesteremia and hyperphospholipemia were observed (Adlersberg *et al.*, 1953 and 1954). Independently, Cook *et al.* (1952) and Gordon *et al.* (1954) observed that cholesterol fed rabbits treated with cortisone exhibited lower levels of serum cholesterol than those receiving cholesterol only. In contrast, Oppenheim and Bruger (1952) and Stumpf and Wilens (1954) found in agreement with our own observations, that the administration of cortisone accentuated hypercholesteremia in the cholesterol fed rabbit. All students of this problem found inhibition of atheroma formation by cortisone in the cholesterol fed rabbit.

This paper deals further with the effects of parenteral administration of corticotropin, cortisone and hydrocortisone on plasma lipid partition of the rabbit maintained on normal rabbit chow with and without cholesterol

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supplements. A similar experimental study was conducted in dogs. The pathological findings will be briefly stated. In the course of this work we became increasingly aware of the importance of tissue permeability upon the development of experimental atherosclerosis. This aspect of the work will be briefly discussed.

PROCEDURE

Rabbits

Two hundred and forty male rabbits, most of them chinchillas, a few albinos and giant checkers, were used in this study. They were about three months of age and weighed 2.0 to 3.0 kg. at the beginning of the experiment. One group of animals was fed Purina pellets and water *ad libitum*. The other group was fed the same Purina pellets pre-treated with cholesterol (Wang *et al.*, 1954). The daily consumption of cholesterol was approximately 1 gm. per rabbit.

Blood was drawn from the marginal ear vein or the central artery, with a drop of heparin solution as an anticoagulant, at intervals of 10 days to two weeks. Total and free plasma cholesterol were determined by the Sperry-Schoenheimer method (1935); phospholipids by Sperry's modification of the Fiske-Subbarow method (1942); and total lipids by Bloor's gravimetric method (1914). Neutral fats were calculated by subtracting the sum of total cholesterol and phospholipids from the figures of total lipids (Ahrens and Kunkel, 1949).

One hundred and fifty rabbits were used to determine the effects of the intramuscular administration of corticotropin, cortisone acetate and hydrocortisone acetate on the lipid partition of the purina chow-fed rabbit. After a control period of 2 to 8 weeks, the animals received daily intramuscular injections of one of the hormones. The mortality encountered in animals which received 3 mg./kg. per day or more of cortisone or hydrocortisone was very high. The daily dosage was therefore reduced subsequently to 1.5–2.5 mg./kg. body weight per day in purina chow-fed animals.

Ninety rabbits were fed cholesterol-treated chow and were given a daily injection of corticotropin, cortisone or hydrocortisone. Since the plan of the study required prolonged duration of these experiments so that sufficient time be given for atherosclerosis to develop, the daily dose of the hormones was further reduced in these experiments to 1.25–1.5 mg./kg. body weight. The injections were started simultaneously with cholesterol feeding in the majority of animals. A group of 28 animals was kept on cholesterol feeding throughout the observation period to serve as controls. In a group of 14 rabbits the experimental procedure was modified in that these animals were first treated with the cholesterol supplement alone; they were then given, in addition to cholesterol, 1.25 mg./kg. of cortisone acetate daily for 2 to 3 months, after which time they were finally placed on a second control period of cholesterol feeding alone.

Dogs

Fourteen young male dogs were used in the study. Their weight varied from 10.5 to 20.8 kg. and averaged 14.4 kg. They were devocalized, immunized against canine distemper and hepatitis and were fed a mixed dog diet of meat, vegetables and milk. Blood was drawn every 2 to 4 weeks in the fasting state. Complete lipid partitions were done as described above. All of the dogs were observed for a control period of one to three months. A three weeks' course of daily intramuscular injections of 100 mg of either corticotropin, cortisone or hydrocortisone was then instituted. After the discontinuation of the hormone administration the animals were observed for another one to two months so that each animal served twice as its own control.

Seven of the 14 dogs were fed 6 gm. cholesterol daily for 120 days. Two of the cholesterol fed animals were injected daily with 100 mg. of cortisone acetate and two with 100 mg. of hydrocortisone acetate for four weeks. Two animals served as controls for cholesterol feeding alone. One animal received corticotropin for two weeks and died; it is not included in the results.

RESULTS

Rabbits

Corticotropin, Cortisone and Hydrocortisone without Cholesterol Supplements. Forty-two rabbits survived hormone administration for ten days or longer (Table 1). The corticotropin-injected animals showed an increase in weight similar to the control animals. The cortisone and hydrocortisone injected animals, however, lost weight as a group, although some of the animals had gained weight in the first one to two weeks of treatment. The single animal of 5.5 kg. which received hydrocortisone for 110 days exhibited a steady moderate decrease of weight.

The most conspicuous alteration occurred in the neutral fat fraction of the plasma (Table 1) which increased from 1.8 times normal under cortico-

TABLE 1. EFFECT OF CORTICOTROPIN, CORTISONE AND HYDROCORTISONE ON PLASMA LIPIDS OF RABBITS

No. of rabbits	Hormone	Body wt., kg.			Daily dose, mg./kg.	Duration, days avg.		Plasma lipid fractions; (mg./100 ml.)				% Est. chol.	P.L./chol.
		Init.	Final	Diff. %				Cholesterol tot./est.	Phospholipid	Neutral fats	Total lipids		
119	None	2.44	2.78	+13.9	0	0	0	49/37	103	198	350	75	2.1
15	Corticotropin	2.03	2.33	+14.8	2.5–4*	15–61	39	95/75	150	337	582	79	1.6
18	Cortisone	1.83	1.52	−16.9	1.5–5	10–60	34	158/89	278	1019	1454	56	1.8
8	Hydro-Cortisone	2.65	2.16	−18.5	1.5–2.5	14–29	21	93/52	187	692	972	56	2.0
1	Hydro-Cortisone	5.50	4.60	−16.4	2.5	110	110	84/32	250	1426	1760	38	3.0

* Expressed as units.

tropin to 7 times normal (seen in the single animal injected with hydrocortisone for 110 days). Marked lactescence was characteristic of these plasmas. The most opaque plasma contained the highest levels of neutral fat. The changes in plasma cholesterol and phospholipids were less pronounced than those in neutral fat. Total cholesterol increased approximately 3 times under cortisone, and approximately 2 times under hydrocortisone and corticotropin. The percentage of esterified cholesterol remained unchanged in the corticotropin treated animals and dropped moderately in the cortisone and hydrocortisone treated animals and considerably in the single animal which received hydrocortisone for 110 days. The phospholipid:cholesterol ratio showed a moderate decrease in the corticotropin and cortisone treated animals; the hydrocortisone treated animals showed no change with the exception of the single animal treated for 110 days which showed an elevation of this ratio. The elevation of total lipids was reflected in all lipid fractions, especially in neutral fat. In 12 rab-

bits the fasting blood sugar was determined. It remained with normal limits during the entire course of the experiment.

Corticotropin, Cortisone and Hydrocortisone with Cholesterol Supplements. The changes in body weights in the various experimental groups are shown in the footnote of Table 2. Cholesterol feeding did not affect the natural gain in weight in this group of young animals. Rabbits injected simultaneously with corticotropin or cortisone showed smaller weight gains than those seen in the former group, while the animals injected with hydrocortisone exhibited a moderate loss of weight.

The effects of cholesterol feeding combined with injections of corticotropin, cortisone and hydrocortisone on plasma lipid fractions were com-

TABLE 2. EFFECT OF CORTICOTROPIN, CORTISONE AND HYDROCORTISONE ON PLASMA LIPIDS OF THE CHOLESTEROL FED RABBIT†

	Hormone	Daily dose, mg./kg.	No. of animal	Time of observations				
				0	2 wks.	4 wks.	6 wks.	8 wks.
Cholesterol total/esters, mg./100 ml.	None	0	28	49/37	330/224	641/458	756/ 568	914/ 669
	Corticotropin	1.5*	4	32/24	320/200	548/403	1164/ 901	1157/ 972
	Cortisone	1.25–1.5	15	54/50	540/420	1056/818	1800/1372	2523/1905
	Hydrocort.	1.25–1.5	10	29/18	579/418	1194/614	1952/1543	1253/ 955
Phospholipids, mg./100 ml.	None	0	28	97	233	330	342	413
	Corticotropin	1.5*	4	99	200	293	498	512
	Cortisone	1.25–1.5	15	100	327	632	684	810
	Hydrocort.	1.25–1.5	10	94	343	520	889	641
Neutral fats, mg./100 ml.	None	0	28	238	778	741	735	893
	Corticotropin	1.5*	4	276	300	782	808	996
	Cortisone	1.25–1.5	15	253	1073	1172	1716	1777
	Hydrocort.	1.25–1.5	10	82	1158	1186	1479	1686
Total lipids mg./100 ml.	None	0	28	384	1341	1712	1833	2220
	Corticotropin	1.5*	4	407	820	1623	2470	2665
	Cortisone	1.25–1.5	15	307	1940	2860	4200	5110
	Hydrocort.	1.25–1.5	10	205	2080	2900	4320	3580

* Expressed as units.
† Body weight of rabbits presented in the above table:

Hormone	Initial weight	Final weight	Difference, %
None	2.70	3.05	+12.6%
Corticotropin	2.74	2.94	+ 7.3%
Cortisone	2.72	2.85	+ 4.8%
Hydrocortisone	2.93	2.82	− 3.8%

pared at intervals of 2, 4, 6 and 8 weeks with those observed in the animals treated with cholesterol solely (Table 2). In each instance the cholesterol-fed and cortisone or hydrocortisone-injected animals had lipid levels markedly higher than those fed cholesterol alone. Corticotropin differed in its effects upon plasma lipid partition from those of cortisone and hydrocortisone. After administration of corticotropin for 2–4 weeks there were no changes in the lipid fractions of the animals. After 6–8 weeks of combined treatment, however, elevation of all lipid fractions was present. The percentage of esterified cholesterol did not change significantly in the various groups except for a moderate elevation in those treated with corticotropin. In the latter the esterified cholesterol fraction rose from the normal level of 75% to 84%. The phospholipid:cholesterol ratio showed a pro-

gressive drop under cholesterol feeding from 2.1 to 0.45 after 8 weeks of cholesterol feeding (Wang *et al.*, 1954). Combination of cholesterol and cortisone resulted in an additional drop of this ratio to 0.32 while corticotropin or hydrocortisone administration did not affect the ratio significantly.

The results observed in 14 animals which were given both cortisone injections and cholesterol feeding between two periods of cholesterol feeding alone are shown in Figure 1. During the first period the animals were fed cholesterol only: 3 animals, 156 days; 3, 53 days; 4, 48 days; 4, 44 days. It is evident that during the second experimental period in which the animals received cortisone and cholesterol there was marked additional increase of all plasma lipid fractions. While the percentage of esterified cholesterol remained practically unchanged, the phospholipid:cholesterol ratio showed an additional drop. After discontinuation of cortisone injections but continuation of cholesterol feeding (third period), the levels of all lipid fractions as well as the ratios returned toward the levels observed at the end of the first period within 10 days. They remained unchanged during the subsequent observation periods which extended up to 92 days.

Dogs

The data in the dogs are summarized in Table 3. As in the rabbit a remarkable constancy of the levels of the lipid fractions for the individual dog was observed in repeated analyses over a period of 1–3 months.

Daily injections of corticotropin, cortisone or hydrocortisone produced very small changes in the lipid fractions. Corticotropin administration resulted in a slight rise in the cholesterol, phospholipid and total lipid fractions, but a drop in the neutral fat fraction. Cortisone or hydrocortisone injections produced an elevation of all plasma lipid fractions. The esterified

TABLE 3. MAXIMAL CHANGES IN PLASMA LIPIDS OBSERVED WITH DIFFERENT EXPERIMENTAL REGIMENS IN THE DOG

	Time in weeks	Cholesterol		Phospholipids		Neutral fats		Total lipids			
		mg./100 ml.	Times normal	mg./100 ml.	Times normal	mg./100 ml.	Times normal	mg./100 ml.	Times normal	% of est. chol.	Ratio P.L./chol.
Control	—	150 (115)*	0	295	0	225	0	671	0	77	1.97
Cortisone or Hydrocortisone alone	3	171 (130)	1.1	312	1.1	247	1.1	730	1.1	76	1.82
Corticotropin only	3	187 (136)	1.2	337	1.1	161	0.7	685	1.0	73	1.80
Cholesterol feeding only	12	234 (184)	1.6	397	1.3	392	1.7	1023	1.5	79	1.70
Cholesterol and Cortisone or Hydrocortisone	4	467 (354)	3.1	485	1.6	663	2.9	1615	2.4	76	1.04

* Figures in parentheses represent esterified cholesterol.

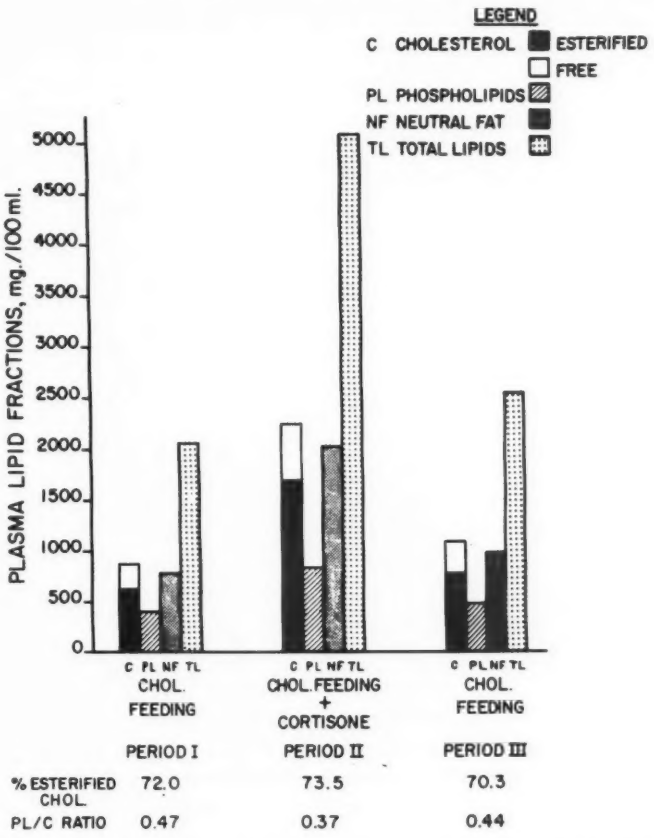

FIG. 1. Effect of daily cortisone injections on the plasma lipids of the cholesterol-fed rabbit. For details, see text.

cholesterol remained unchanged, while the phospholipid:cholesterol ratio showed a moderate drop.

Cholesterol feeding in daily doses of 6 gm. over a period of 120 days produced elevations of all lipid fractions but not nearly of the magnitude of those observed in the rabbit under similar conditions. The ratio of esterified cholesterol remained unchanged; the phospholipid:cholesterol ratio fell moderately.

The combination of cholesterol feeding with daily injections of cortisone or hydrocortisone in doses of 100 mg. for 30 days produced a significant elevation of all plasma lipid fractions and resulted in the highest figures observed in this series. The esterified plasma cholesterol remained unchanged. The phospholipid:cholesterol ratio showed a marked drop of approximately 50%. Cessation of hormone injections but continuation of

cholesterol feeding resulted in a drop from the highest levels of plasma lipids noted toward those which had been previously observed on cholesterol feeding alone.

Pathological Findings in the Rabbit

In all rabbits careful pathological studies were performed. With one exception none of the 42 rabbits treated on regular chow with corticotropin, cortisone or hydrocortisone developed atherosclerosis. The exception was a rabbit treated with hydrocortisone for 110 days. Small elevated areas of atheroma, discrete and confluent, were found over the ascending aorta; two tiny plaques were seen in the aortic arch and several small areas in the pulmonary artery. It must be stressed that this was the heaviest animal of the group; it tolerated hydrocortisone administration for almost 4 months. Despite the negative findings in the other animals the possibility cannot be ruled out that prolonged hormonal administration on regular diets may result in experimental atheroma production. Additional studies, however, are necessary to prove this possibility.

After one month of administration of 1 gm. of cholesterol per day tiny but definitely elevated yellowish white plaques of atheroma were found in the ascending aorta and the aortic arch, especially around the orifices of the branching arteries. A few discrete atheromatous plaques were noted in the pulmonary artery, especially at the bifurcation of the right and the left branches.

After 2 months of cholesterol feeding the atheromatous areas of the ascending aorta became larger, more numerous and confluent, and formed irregularly shaped, well demarcated plaques. At this time some of the plaques showed longitudinal orientation. In the descending aorta only discrete small plaques were found. Similar small plaques of atheroma were regularly seen in the branches of the pulmonary artery and in the aortic or mitral valves.

After 3–4 months severe atherosclerosis was observed; large, thick and confluent plaques covered the ascending and descending aorta; there was considerable thickening and atheromatosis of the mitral and/or aortic valves, and of the coronary and pulmonary arteries. Lipidosis of kidneys and spleen was usually present. Some animals exhibited xanthomata of the skin and joints.

Animals injected with cortisone or hydrocortisone in addition to cholesterol feeding exhibited at comparable time intervals milder degrees of atherosclerosis, despite higher plasma lipid levels, than those observed in the animals receiving cholesterol supplements alone. The atheromatous lesions were less extensive and confluent, were smaller and thinner, and only rarely involved the descending aorta. Similarly the changes in the pulmonary artery and in the mitral or aortic valves were of a milder degree. In some animals of the series practically no atheromatous lesions were

observed after 45 days of the experiment. A summary of the pathological changes is presented in Table 4.

DISCUSSION

The administration of corticotropin, cortisone and hydrocortisone produced increases in all plasma lipid fractions in the rabbit. Since regular Purina chow contains no cholesterol (our own analysis) the increase in plasma lipids is endogenous in origin. Best *et al.* (1936) demonstrated the mobilization of body fat in mice injected with anterior pituitary extracts. Stoerck (1950) noted prevention of loss of body fat in the adrenalectomized animal by the administration of cortisone. Rich *et al.* (1951) observed lipemia and a decrease of body adipose tissue in rabbits treated with

TABLE 4. EFFECT OF HORMONAL INJECTION ON THE DEVELOPMENT OF ATHEROSCLEROSIS IN CHOLESTEROL-FED RABBITS

(Duration of Experiment: 6–8 weeks)

Procedure	Ascending aorta	Descending aorta	Pulmonary arteries	Mitral and/or aortic valves
Chol. feeding	++	+	++	+
Chol. feeding +Cortisone	±–+	±	+	0–±
Chol. feeding +Hydrocortisone	±–+	±	+	0–±

cortisone injections and suggested that cortisone mobilized body fat. It should not be overlooked that the occurrence of infection and toxicity in rats injected with large doses of cortisone (Antopol, 1950), may *per se* result in decrease in body weight and adipose tissue. We observed marked signs of toxicity in rabbits receiving large doses of this hormone. The food consumption of these animals decreased, the fur became shaggy and ruffled; rapid loss of weight was associated with muscular weakness and atrophy; severe infections such as conjunctivitis, keratitis, pneumonia, pleurisy, pericarditis, and abscess formation complicated the picture. Marked fall of esterified plasma cholesterol was invariably noted, probably indicating hepatic damage. These observations prompted us to reduce considerably the daily dose of the hormone (see procedure).

The studies of Pierce and Bloom (1952) suggested that cortisone produced, at least in part, a metabolic block at the lipoprotein levels of Sf 40–80. Their experimental technique is however open to criticism because daily large blood specimens were drawn for the determination of serum lipids and lipoproteins, a procedure which may in itself produce plasma lipid changes (Hoffmeyer 1944).

The results of our studies on the effect of administration of hormones on plasma lipids of the rabbit fed regular chow are in agreement with our own

previous observations, as well as those of others. The observations in the cholesterol-fed rabbit treated with corticotropin, cortisone and hydrocortisone are in agreement with the results of some authors (Oppenheim and Bruger, 1952; Adlersberg *et al.*, 1953, 1954; Stumpf and Wilens, 1954) but in disagreement with others (Cook *et al.*, 1952 and Gordon *et al.*, 1954). Our experience showed that rabbits receiving cortisone in doses of 3 mg./kg. or more exhibited toxic manifestations. Long-term experimentation even with small doses of the hormone sometimes produced evidences of toxicity such as considerable loss of weight and muscular atrophy, and was then associated with a drastic fall of all plasma lipid fractions. This is illustrated by the behavior of the hydrocortisone group (Table 2, lines 4 of the corresponding categories), during the last two weeks of the experiment. It is important in these experiments to register continuously changes in body weight, in general condition and in esterified cholesterol in order to detect early manifestations of toxicity. With prolonged use of the hormones in moderate but adequate doses and in carefully conducted experiments decided enhancement of hypercholesteremia and the other lipid fractions in the cholesterol fed rabbits was the rule.

The effects of these hormones were also studied in the chick (Stamler, Pick, and Katz, 1951; Paterson *et al.*, 1951; Stamler *et al.*, 1952). In evaluating these findings one must not forget that the lipid metabolism and the metabolic effects of corticosteroids in birds differ from those in mammals.

Since Anitschkow (1913) it has been well established that both the plasma cholesterol concentration and the duration of hypercholesteremia are the main determining factors for the development of experimental atherosclerosis. The observation that the hormone-treated cholesterol-fed rabbit showed less atherosclerosis than the cholesterol-fed animals, despite enhanced hypercholesteremia, suggests that factors other than concentration and time exert a decisive influence in atherosclerosis. Stumpf and Wilens (1954) believe that the relative proportion of cholesterol and other serum lipid fractions is of significance in atherogenesis. Decreased tissue permeability as a result of cortisone or hydrocortisone administration offers, in our opinion, a plausible explanation. Seifter *et al.* (1953) observed, despite lower plasma cholesterol levels, enhancement of atherogenesis in cholesterol-fed rabbits injected with hyaluronidase. He explained his results on the basis that hyaluronidase lowered hypercholesteremia by increasing the rate of cholesterol deposition in the tissues. Benditt *et al.* (1950) demonstrated that corticosteroids inhibited the effect of hyaluronidase on capillary permeability in rats. A possible protective action against atherogenesis, through the mechanism of decreased tissue permeability, may be indicated by the finding of diminished atherosclerosis as well as diminished deposition of cholesterol in other tissues in cholesterol fed hormone-treated rabbits. The role of the ground substance and its perme-

ability in the evolution of experimental atherosclerosis is now under study (Wang *et al.*, 1954).

The injection of cortisone or hydrocortisone in the cholesterol-fed dog produced serum lipid levels which have previously been obtained only by cholesterol feeding combined with thyroidectomy, or by feeding cholesterol in amounts at least twice as great as those used by us. This raised the possibility of the corticosteroids acting, at least in part, through depression of thyroid function. Observations pertaining to this question will be presented elsewhere.

SUMMARY

Rabbits treated with cortisone and hydrocortisone exhibited marked opacity of the plasma and significant elevations of all plasma lipids fractions, but especially of neutral fats and total lipids.

The combined administration of these hormones and cholesterol feeding resulted in enhanced elevations of all lipid fractions when compared with the results obtained by cholesterol feeding solely. The plasma of these animals had the appearance of cream, and its turbidity was much greater than that of the other groups treated with cortisone or hydrocortisone alone or with cholesterol supplements solely.

Despite extreme elevation of all lipid fractions the cholesterol-fed hormone-injected animals exhibited less atherosclerosis than those treated with cholesterol alone. Hormonally induced diminished permeability of the tissue offers a plausible explanation of the experimental findings.

Although the combination of cholesterol feeding with corticosteroids significantly enhanced the elevations of all lipid fractions of the dog, these changes fell far behind those seen in the rabbit.

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INCORPORATION OF RADIOACTIVE PHOSPHORUS INTO THE NUCLEIC ACIDS OF SURVIVING RAT UTERI: EFFECTS OF STEROID HORMONES *IN VITRO* AND *IN VIVO*[1]

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INTRODUCTION

MARKED alterations in uterine growth and physiology occur under the stimulation of estrogenic and androgenic steroids. On the assumption that desoxypentose and pentose nucleic acids (DNA and PNA) play an important part in these responses, an investigation was made in this laboratory into the effect of such steroids on the purine-pyrimidine composition of DNA and PNA extracted from uterine tissue. We have reported that compositional variations were apparently associated with the response of human and animal preparations to these steroids (Gold and Sturgis, 1954). The purpose of our present study was to determine the ability of steroid hormones to alter the rate of synthesis of uterine nucleic acids *in vitro*. The incorporation of P^{32} has been employed as an index of this synthesis. Changes in rate of incorporation associated with exposure to steroids either *in vivo* or *in vitro* may suggest one of the mechanisms whereby these hormones may bring about their profound effects on uterine growth and differentiation.

MATERIALS AND METHODS

Preparation of tissue. Wistar strain mature female rats weighing 80 to 120 gm. were used throughout. All animals were first castrated to eliminate cyclic fluctuations in endogenous ovarian steroids and one month after ovariectomy a group of these furnished uteri labeled "castrate" in these experiments. The two experimental uterine preparations were developed with estrogen injections alone and with estrogen plus progesterone according to standard specifications previously detailed to produce uteri herein labeled "estrogen" and "deciduoma" respectively. In order to emphasize the difference in hormone regime, it may be pointed out that the "estrogen" rats received 2 μg. estradiol dipropionate in sesame oil daily for 3 days prior to sacrifice, while the "deciduoma" rats received 1.5 mg. progesterone in sesame oil for 7 days before sacrifice after prior estrogen priming and traumatization. Purina chow and water were given *ad libitum*. The tissues were removed immediately after sacrifice. The "castrate" uteri were trimmed, then

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minced[2] in the cold; the "estrogen" uteri were minced after expressing the intra-uterine fluid; the "deciduoma" tissue was minced after stripping the decidua from the myometrial wall.

In vitro system. Approximately 200 to 300 mg. of minced tissue was placed in 3 ml. of a Krebs-Ringer-bicarbonate buffer, initial pH 7.4, containing 25 μc. P^{32} as PO_4. This flask was shaken at 37° in an atmopshere of air for 2 or 3 hours. The reaction was stopped by the addition of 7% trichloracetic acid (TCA) in the cold.

In vitro addition of steroids. On the day before each experiment crystalline steroids were dissolved in ethanol and 500 μg. aliquots were pipetted into the incubation flasks. The alcohol was evaporated leaving a film of steroid upon the bottom of the flask. Evaporated alcohol controls were used.

In vivo administration of steroids. Intramuscular injections of steroid in 0.1 to 0.2 ml. of sesame oil were given as indicated.

Phosphorus fractions. The method employed for the preparation of the total nucleic acids (TNA) was essentially that of Schneider (1945). The incubation flask contents was washed with 4 vol. cold 7% TCA homogenized in 10–20 volumes of cold 7% TCA, washed 2 times with 30–40 volumes cold 7% TCA, extracted twice at room temperature with 30–40 volumes of 3:1 ethanol-ether to remove phospholipids, and the residue extracted twice with 5% TCA at 90° to yield the TNA fraction. In certain experiments the phospholipids removed by alcohol-ether extraction at room temperature were assayed for their specific activity.

Phosphorus analyses were performed by digestion of the fraction with hot sulfuric acid and hydrogen peroxide followed by a Fiske-Subbarow (1925) determination of inorganic phosphorus. In a few instances the method of Schmidt and Thannhauser (1945) was used to prepare the pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) fractions.

Radioactive measurements. All counting was done in duplicate to 2% error. An end-window Geiger counter and an Argon flow counter were employed. Corrections for coincidence and self-absorption were applied.

Calculations. The specific activity (s.a.) of the fractions is expressed as the counts per minute per mg. phosphorus. This s.a. is compared to the s.a. of the control (steroid omitted) from the same run.

The level of specific activities derived from each experiment depends basically on the radioactivity of the media on each day. The level of s.a. in one experiment cannot be compared with that obtained in another experiment on another day.

RESULTS

The rapid uterine growth after traumatization is indicated in Figure 1. The increment in wet weight per day of the deciduomata is of course largely water, but it is easy to see that the water uptake is accompanied as well by an increase in dry weight components, i.e. there is a daily increase in non-aqueous protoplasmic constituents.[3] Figure 1 also shows that with the growth of the deciduoma there is an apparent increase in the ability of the tissue to incorporate radioactive phosphorus into the nucleic acid fraction.

[2] Minces were prepared by cutting the tissue with iris scissors into approximately 1–3 cu. mm. sections. Homogenates were not employed.

[3] Our data show that the deciduomata are approximately 16% dry weight. If the daily wet weight increase is 150–200 mg. then the corresponding dry weight increment is 24-32 mg.

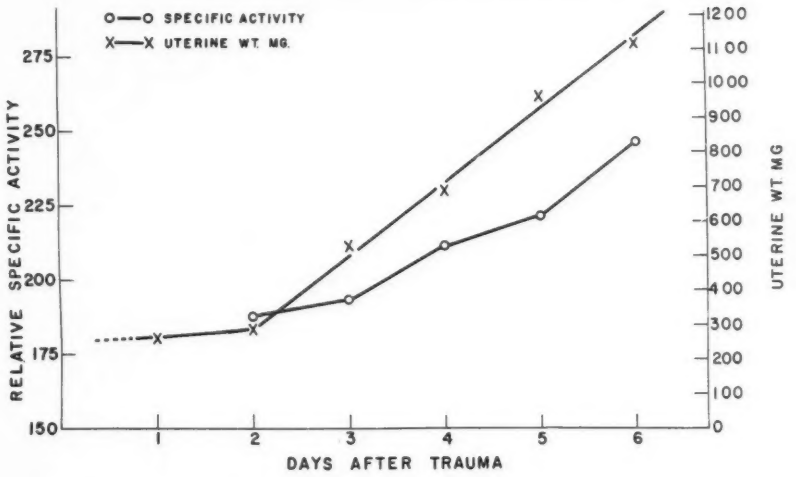

FIG. 1. *In vitro* uptake of P^{32} by nucleic acids of pooled rat uterine deciduoma of varying age. (See test for explanation.)

There is an increase in the rate of incorporation of radioactive phosphorus into the TNA of deciduomata, in relation to duration of exposure to the isotope in the incubating flasks. Figure 2 indicates that this rate is still increasing at 6 hours of incubation and that there is an almost complete inhibition of the uptake by both cyanide and dinitrophenol.

The presence of a dry film of certain of the steroids on the bottom of the flask consistently suppresses this incorporation. Table 1 indicates that under these experimental conditions dehydroepiandrosterone (DHA), testosterone, ethyl testosterone, and progesterone all inhibit the P^{32} uptake into deciduoma nucleic acids. Similar inhibitory effects are observed in the case of the "estrogen" uteri as shown in Table 2. It will be noticed, however, in Tables 1 and 2 that several steroids do not produce the inhibitory effect. Thus, with cholesterol, pregnanediol, and 17 hydroxycorticosterone (Kendall's Compound F) there is no significant difference between the controls and the incubations containing steroid.

The inhibitory effect of DHA upon the P^{32} uptake into decidual TNA, shown in Table 1, does not appear to be specific for one type of nucleic acid. The incorporation of P^{32} into both DNA and PNA of "deciduomata" was depressed 50% by the *in vitro* film of DHA. This was observed in a series of 28 incubations using samples from a "pool" of uterine tissue obtained from 18 rats.

Steroid films added to the flasks do not act exclusively on the synthesis of nucleic acids. The incorporation of P^{32} into the "deciduoma" phospholipids (Table 3) is also diminished by the presence of *in vitro* films of DHA, testosterone, and ethyl testosterone.

TABLE 1. *In vitro* EFFECT OF STEROIDS ON THE P^{32} INCORPORATION INTO TOTAL NUCLEIC ACIDS OF RAT DECIDUOMATA

Steroid	No. of rats	No. of incubations	Specific activity[1]		Inhibition	P†
			Control	With steroids*		
Dehydroepiandrosterone	21	10	67 ± 2	19 ± 1	72%	0.001
Dehydroepiandrosterone	28	9	71 ± 3	32 ± 4	55%	0.001
Dehydroepiandrosterone	17	8	62 ± 3	19 ± 2	67%	0.001
Dehydroepiandrosterone	24	9	270 ± 8	212 ± 10	22%	0.001
Dehydroepiandrosterone	21	12	64 ± 1	32 ± 2	50%	0.001
Testosterone	16	15	87 ± 3	60 ± 2	31%	0.001
Testosterone	21	10	67 ± 2	52 ± 4	22%	0.007
Testosterone	28	9	71 ± 3	60 ± 3	15%	0.025
Ethyl Testosterone	21	10	67 ± 2	30 ± 5	55%	0.001
Ethyl Testosterone	28	9	71 ± 3	52 ± 4	27%	0.004
Progesterone	17	10	179 ± 8	122 ± 10	32%	0.002
Cholesterol	19	13	372 ± 26	338 ± 34	—	0.5
Pregnanediol	8	6	120 ± 6	173 ± 29	—	0.2
Pregnanediol	9	6	194 ± 12	191 ± 13	—	—
Pregnanediol	5	10	286 ± 27	306 ± 20	—	0.6
Desoxycorticosterone	17	10	179 ± 8	159 ± 9	—	0.13
Cortisone, (Kendall's Compound E)	19	13	372 ± 26	305 ± 19	—	0.06
17 hydroxycorticosterone (Kendall's Compound F)	17	10	179 ± 8	161 ± 15	—	0.6

[1] Mean values with standard errors expressed as counts per minute per mg. total nucleic acid phosphorus.

* A dry film of 500 μg. steroid was plated out on the bottom of each vessel prior to incubation in all experiments, Tables 1–4.

† P is the Probability obtained from a Fisher "t" curve, for the significance between the two means. $P > 0.05$ indicates no significant difference.

TABLE 2. *In vitro* EFFECT OF STEROIDS ON THE P^{32} INCORPORATION INTO TOTAL NUCLEIC ACIDS OF RAT "ESTROGEN" UTERI*

Steroid	No. of rats	No. of incubations	Specific activity		Inhibition of uptake	P
			Control	With steroid		
Dehydroepiandrosterone	21	8	45 ± 2	19 ± 2	58%	0.001
Testosterone	21	8	45 ± 2	33 ± 1	27%	0.002
Progesterone	26	10	285 ± 10	201 ± 13	29%	0.001
Cholesterol	26	10	285 ± 10	288 ± 13	—	—
Pregnanediol	21	8	45 ± 2	45 ± 2	—	—
17 hydroxycorticosterone	26	10	285 ± 10	262 ± 13	—	—

* See Table 1 footnotes.

TABLE 3. *In vitro* EFFECTS OF STEROIDS ON P^{32} INCORPORATION INTO PHOSPHOLIPIDS OF RAT DECIDUOMATA

Steroid	No. of rats	No. of incubations	Specific activity*		Inhibition of uptake	P
			Control	With steroid		
Dehydroepiandrosterone	21	10	78 ± 4	24 ± 2	69%	0.001
Dehydroepiandrosterone	17	8	112 ± 3	37 ± 2	67%	0.001
Testosterone	16	16	136 ± 6	93 ± 2	32%	0.001
Testosterone	18	9	78 ± 4	74 ± 3	—	—
Ethyl Testosterone	18	10	78 ± 4	19 ± 3	76%	0.001

* Mean values with standard errors expressed as counts per minute per mg. lipid phosphorus. See Table 1 footnotes.

The effects noted in Tables 1 to 3 were obtained from shaking the tissues, isotope, and media for a few hours in flasks coated with a thin film of a known amount of relatively insoluble steroids. The concentration of these substances actually available to the tissue minces and responsible for these effects could not be estimated. Solutions of known concentrations of the steroids were therefore prepared by placing an excess of the compouud in boiling distilled water, cooling, and filtering free of solids. The amounts

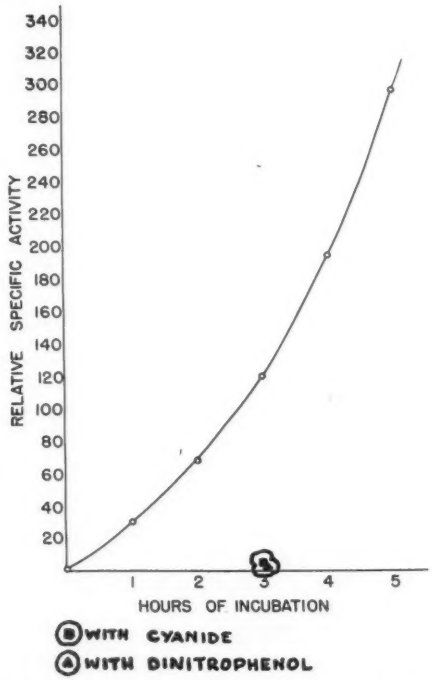

FIG. 2. *In vitro* uptake of P^{32} by nucleic acids of pooled 5 day rat uterine deciduomata.

TABLE 4. *In vitro* EFFECTS OF KNOWN CONCENTRATIONS OF STEROIDS IN SOLUTION ON P^{32} INCORPORATION INTO TOTAL NUCLEIC ACIDS OF RAT UTERI

Type of uterus	Steroid solution[c]	μg. Steroid per ml. media (measured)	Specific Activity		Inhibition of uptake
			Control	With steroid	
Deciduoma	Dehydroepiandrosterone	11[a]	232	241	None
Deciduoma	Testosterone	26–78[b]	220	211	None
Deciduoma	Progesterone	11[b]	232	225	None
Deciduoma	Progesterone	11[b]	220	214	None
Estrogen	Progesterone	11[b]	55	52	None

[a] Determined by a Zimmermann color reaction on the filtered steroid solution prior to preparation of the incubation media.
[b] Determined by ultra-violet absorption of the filtered steroid by comparison with known standards.
[c] See Text.

in solution were determined by absorption in the ultra violet or by the Zimmermann (1935) reaction. Table 4 shows that such preparations containing from 11 to 78 μg. of steroid per incubation flask had no effect on the P^{32} uptake into rat uterine nucleic acids.

Testosterone was then investigated to compare the effects of its administration *in vivo* with those previously shown *in vitro*. The same incubation system was employed. The *in vivo* results with testosterone show some marked differences from the *in vitro* action of the hormone. In the "deciduoma," Table 5, testosterone in a range of 2 μg. to 2 mg. each day for 3 days prior to sacrifice caused a clear-cut depression in nucleic acid

TABLE 5. *In vivo* EFFECT OF TESTOSTERONE ON P^{32} INCORPORATION INTO TOTAL NUCLEIC ACIDS OF RAT UTERI

Type of uterus	Testosterone dose*	No. of rats	No. of incubations	Specific activity†		Per cent change	P
				Control	With steroid		
Deciduoma	a	12	13	226 ± 8	176 ± 9	− 22%	0.002
Deciduoma	b	13	11	234 ± 9	177 ± 13	− 24%	0.006
Deciduoma	c	18	10	225 ± 12	88 ± 3	− 61%	0.001
"Estrogen"	b	9	5	189	249	+ 32%	—
"Estrogen"	b	22	10	55 ± 3	98 ± 4	+ 78%	0.001
"Estrogen"	c	14	10	99 ± 2	155 ± 9	+ 57%	0.001
"Estrogen"	c	17	10	34 ± 2	113 ± 2	+231%	0.001
"Estrogen"	d	24	10	55 ± 2	55 ± 4	0	—
Castrate	c	40	10	294 ± 10	246 ± 9	− 12%	0.006

* a. 2 mg. testosterone in 0.1 ml. sesame oil injected intramuscularly (IM) each day for 3 days prior to sacrifice.
b. 1 mg. testosterone injected similarly.
c. 2 μg. testosterone injected similarly.
d. 1 mg. testosterone in 0.1 ml. sesame oil injected IM 24 hours prior to sacrifice.
† See Table 1 footnotes.

"synthesis." In the "estrogen" uterus the same dose of testosterone stimulated P^{32} uptake into the nucleic acids. When, however, the testosterone was administered to the "estrogen" rats only 24 hours before sacrifice, then no effect upon P^{32} incorporation was observed.

A comparison of the *in vivo* action of testosterone upon the P^{32} incorporation into nucleic acids of several organs other than the uterus appears in Table 6. As noted in Table 5, uterine uptake was markedly depressed in the deciduoma rat and stimulated in the estrogen rat. Effects were also noted on the uptake into liver, spleen and kidney nucleic acids from the same animals but the order of magnitude of either stimulation or depression resulting from the action of this androgen on non-target organs was apparently less marked, although in some instances statistically significant. It is further of interest that where such non-target organ effects were observed they were in the same range as those obtained in the castrate uterus.

DISCUSSION

The incorporation of radioactive phosphate *in vitro* into uterine nucleic acids offers one system to study the effects of combinations of steroids on nucleic acid synthesis. In this system the "biologically active" sex steroids, testosterone and progesterone, when present as films are effective in depressing the incorporation of P^{32}, but DHA, a compound with little or no known biologic effect on the uterus had a similar effect in both deciduomata and estrogen-stimulated uteri. However, other steroids not generally considered to be "biologically active" with respect to the uterus, were ineffective in this system (Tables 1 and 2).

In vitro testosterone and DHA do not specifically affect nucleic acid synthesis alone. In fact these steroids produce similar alterations in phospholipid synthesis also (Table 3). These inhibitory effects may be the result of the repression of some reactions common to nucleic acid turnover and phospholipid turnover. Others (Dirscherl *et al.*, 1952; Dorfman and Goldsmith, 1951; Meyer, 1952) have pointed out that several of the steroids *in vitro* may inhibit a variety of enzymatic processes. Hayano *et al.* (1950) have shown an inhibition of d-amino acid oxidase by desoxycorticosterone (DOC). Hauschildt and Grossman (1953) demonstrated that testosterone, estradiol, and DOC depress the incorporation of labelled glycine into rat liver protein and Bern (1953) showed that testosterone and estradiol can inhibit the rate of oxygen consumption of the rat ventral prostate.[4]

It may be that the degree of inhibition recorded in these experiments

[4] A notable exception to these inhibitory effects is the finding by Villee and Hagerman (1953) that estradiol-17-B added *in vitro* accelerates the oxidative metabolism of placental tissue.

relates primarily to an "unphysiologic" concentration of steroid to which the tissues were exposed in the incubating flasks. When our preparations were exposed to relatively large quantities (500 μg.) of only slightly soluble steroids coating the bottom of the flasks, we could not estimate how much of these substances may have been available to the tissues. Concentrations of 11 μg. per ml. of DHA and progesterone (0.0001%) and 26 μg. per ml. of testosterone (0.003%) failed to elicit any response (Table 4) and it appeared unlikely that even weaker dilutions would have any different result.

Our findings after giving the sex steroids *in vivo* differed greatly from those here reported after the *in vitro* addition of steroid. An arbitrary dose ranging from 2 μg. to 2 mg. per day for the 3 days before sacrifice was selected. Again it is impossible to estimate the steroid concentration in the uterus itself, but it was shown that as little as 6 μg. of testosterone per 200 gm. rat was sufficient to depress the P^{32} incorporation into uterine deciduomata (Table 5), while 22 μg. per ml. of the same steroid *in vitro* had no effect (Table 4).

In "estrogen" uteri there was a sharp contrast between the *in vitro* and *in vivo* effects of testosterone. Whereas the testosterone film *in vitro* inhibited the uptake of P^{32} by uterine nucleic acid and 22 μg. per ml. of the steroid had no effect, the *in vivo* experiments demonstrated an acceleration of this uptake even with a total of only 6μg. of testosterone over 3 days to a 150–200 gm. animal (Table 5). These data suggest different modes of action for testosterone *in vivo* and *in vitro* in this system.

Others have recorded experiments relating nucleic acid synthesis to steroid hormone activity in a variety of tissues. Thus, Grauer *et al.* (1950) have demonstrated the increased incorporation of P^{32} into the acid insoluble fraction of the uterus of the rat under estrogen priming; Symeonidis and Loustalot (1953) have reported deciduoma formation in mice to be related to the incidence of mammary tumor; Rabinovitch et al. (1951) has shown that testosterone propionate can alter the nucleic acid phosphorus content of rat seminal vesicles; Kelly *et al.* (1950, 1951) have reported an increased rate of incorporation of P^{32} into the DNA of livers and spleens in tumor-bearing mice and rats and in pregnant mice and rats as compared to "normal" animals.

Testosterone *in vivo* appears to be only relatively specific for the target organ tissues. Although this compound did affect the rate of incorporation into nucleic acids of liver and spleen, this was in a range far below that obtained in the "estrogen" and "deciduomata" uterus (Table 6). In this experiment the castrate uterus showed the same level of response as that of non-reproductive tract tissues.

The present experiments lend support to the broad thesis that the effect of administered androgens and estrogens on target organs is conditioned by the previous hormonal environment of such tissues (Courrier, 1950;

TABLE 6. *In vivo* EFFECT OF TESTOSTERONE ON P^{32} INCORPORATION INTO TOTAL NUCLEIC ACIDS OF SEVERAL TISSUES

Tissue	Testosterone dose*	No. of rats	No. of incubations	Specific activity†		Per cent change	P
				Control	With steroid		
Deciduoma rat							
Uterus	c	18	10	225 ± 12	88 ± 3	− 61%	0.001
Liver	c	18	10	48 ± 2	48 ± 4	—	—
Spleen	c	18	10	28 ± 0.8	32 ± 1	+ 16%	0.011
Estrogen rat							
Uterus	c	17	10	34 ± 2	113 ± 2	+231%	0.001
Liver	c	17	10	59 ± 2	48 ± 3	− 12%	0.011
Kidney	c	17	9	90 ± 12	81 ± 7	—	—
Castrate rat							
Uterus	c	40	10	294 ± 10	246 ± 9	− 12%	0.006
Liver	c	40	10	115 ± 3	135 ± 2	+ 12%	0.0015
Spleen	c	40	10	84	84	—	—

* 2 μg. testosterone in 0.1 ml sesame oil injected 1M each day for 3 days prior to sacrifice.
† See Table 1 footnotes.

Roberts and Szego, 1953). Since the rate of synthesis of nucleic acids probably bears a close relationship to cell growth and proliferation, it appears that such studies of the effect of specific steroids on P^{32} incorporation into the nucleic acids of tissues may eventually clarify some of the problems of malignant as well as benign growth responses in the reproductive organs.

SUMMARY

The incorporation of radioactive phosphorus, as phosphate, into the nucleic acids of the castrate, estrogen and deciduoma uterus of the rat, has been observed in an *in vitro* system.

In the deciduomata, P^{32} incorporation is depressed by the presence of a film of dehydroepiandrosterone (DHA), testosterone, ethyl testosterone and progesterone, while the similar addition of cholesterol, pregnanediol, desoxycorticosterone, cortisone or hydrocortisone showed no effect.

DHA, testosterone and progesterone, *in vitro*, had a similar effect on the estrogen uteri, and again cholesterol, pregnanediol and hydrocortisone were ineffective.

P^{32} incorporation into deciduomata *phospholipid* was also inhibited by the *in vitro* addition of a film of DHA, testosterone and ethyl testosterone.

When solutions ranging from 11 to 78 μg. per ml. of the inhibitory steroids were present instead of the films no inhibition was observed.

Testosterone, given *in vivo* to the deciduoma rat inhibits the *in vitro* incorporation of P^{32} into the uterine nucleic acids, whereas similar administration of this steroid to the estrogen rat stimulates the incorporation. Under these same conditions testosterone has a slight depressive effect in the castrate rat.

Spleen, liver and kidney do not respond as markedly as does the uterine tissue to testosterone given *in vivo*.

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STEROIDOGENESIS BY INTACT CALF ADRENALS PERFUSED *IN VITRO*

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INTRODUCTION

THE biogenesis and secretion of corticoids by isolated adrenal glands perfused with homologous whole blood has been reported by Pincus *et al.* (1951) and Vogt (1951). The principal objectives of the present investigation were to establish relatively simple and defined experimental conditions under which steroidogenesis could be studied in intact calf adrenals perfused *in vitro*, and to modify these fundamental conditions in order to ascertain whether the adrenal cortex can adapt its secretory activity and respond directly to the alterations.

Although the hypophyseal discharge of ACTH following a variety of stresses is generally recognized as an essential step in the series of events, which lead to marked acceleration of adrenocortical function, evidence has been adduced in support of the thesis that the cortical cells possess some autonomy and can respond independently of corticotrophic release to alterations in the "milieu intérieur" induced by stressful conditions. It is obvious that the use of homologous blood for the perfusion of isolated adrenals precludes a direct approach to this problem because of its variability and the multiplicity of its constituents, several of which are believed to play a regulatory role in corticoid production and release.

Preliminary experiments demonstrated that intact calf adrenals, perfused *in vitro* with an oxygenated, isotonic artificial medium containing only balanced inorganic salts and glucose, biosynthesized and secreted corticoids, and that this steroidal output could be augmented severalfold by the addition of ACTH to the medium. The comparative secretory activity of adrenals perfused with this artificial medium and with homologous whole blood was investigated. Employing the artificial perfusation fluid, studies were conducted of the acute, direct effect exerted by oxygen, ACTH and other hormones, and various physiological inorganic and organic substances on adrenocortical activity.

MATERIALS AND METHODS

Weighed calf adrenals, varying from 2.5 to 4.5 gm., were excised at a local slaughterhouse, dissected free of periadrenal fat and connective tissue, and kept in an ice-cold

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[1] The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

citrate-saline solution until cannulated and perfused via the adrenal vein according to the technique of Hechter *et al.* (1953). A non-pulsatile hydrostatic pressure of 75 mm. Hg. was employed. Prior to mounting in the apparatus, the glands were perfused with an isotonic citrate-saline solution for four minutes to remove residual blood and detect leaks which required tying. Parallel banks of two left and two right adrenals and the four contralateral control glands were simultaneously perfused in individual multicycle systems within an insulated room, thermostatically maintained at 37.5° C. The perfusion medium was either an artificial fluid or homologous citrated whole blood. The latter prior to adrenal passage was perfused through isolated rat livers to remove foreign matter, thrombi, vasoconstrictors, and other undesirable substances. The artificial perfusion medium (APM) was a glucose-fortified, physiologically balanced and buffered salt solution of the following composition, in grams per liter: glucose 2.00, NaCl 8.2, KCl 0.25, CaCl 0.25, $MgSO_4$ 0.13, $NaHCO_3$ 0.60, NaH_2PO_4 0.05. The blood and artificial media were gassed with a continuous stream of water-filtered 95% O_2/5% CO_2. Preliminary gassing of the artificial fluid for 20 minutes saturated it with oxygen and reduced the pH from 7.7 to 7.4, at which level it remained fairly constant during the experimental period. Penicillin G (20,000 units/L.) and streptomycin (0.1 gm./L.) were added to the freshly-prepared media before perfusion to ensure bacteriostasis. At the end of each experiment, the adrenal effluents were gassed with nitrogen and kept in a frozen state until extracted for corticoid assay.

All the solvents used for the extraction and estimation of corticoids in the perfusates were freshly redistilled. Duplicate or triplicate 100-ml. samples of the thawed APM adrenal effluents were extracted serially with three 100-ml. portions of chloroform. In the case of blood effluents, 15 gm. of NaCl were added to the 100-ml. thawed samples. They were then divided into three portions, each of which was extracted serially with 100, 50, and 50 ml. of chloroform. The combined chloroform extracts of each sample were evaporated to dryness *in vacuo* below 50° C. The residue, dissolved in 5 ml. of chloroform, was chromatographed on florosil columns pretreated with 20 ml. of chloroform, and fractionated by elution with 25 ml. of chloroform, 25 ml. of 2% methanol in chloroform, and 45 ml. of 25% methanol in chloroform, according to the procedure of Nelson and Samuels (1952). The last fraction, containing the relatively uncontaminated corticoid portion, was taken to dryness in a 50° C. water bath with a stream of nitrogen, and the resulting residue dissolved in methanol.

Aliquots of this methanol extract were employed for quantitative estimation of corticoids by three independent methods: ultraviolet absorption at 240 mμ (UV), the Porter and Silber (1950) phenlhydrazine-sulfuric acid reaction for 17, 21-dihydroxy-20-ketones (PS), and the formaldehyde formation from formaldehydogenic steroids (FS), following periodic acid oxidation (Hollander *et al.*, 1951). The values given for each method were derived from reference curves obtained by treating known amounts of cortisone in the same way. At least two standards were run during every analysis of unknowns to determine the accuracy of the recovery procedure. The percentage recovery of cortisone added to APM or whole blood and carried through the entire procedure of extraction, chromatography, etc. averaged better than 85% in each of the three methods employed. All the UV, PS, and FS corticoid values shown represent the means of at least duplicate or triplicate analyses, and are expressed in terms of cortisone equivalents. A correction for non-steroidal impurities as well as possible interfering steroidal substances such as 17-OH progesterone was unnecessary because of the purity of the corticoid fraction following florosil chromatography and the relatively large amounts of corticoids measured. When extracts following florosil chromatography were fractionated by paper partition chromatography and the F, E and B zones eluted and measured, these three corticosteroids together constituted approximately 90% of the total, as de-

termined by UV analysis of the unfractionated extracts. In fact, the latter yielded absorption curves for the UV and PS assays comparable to those obtained with pure compounds.

Total tissue cholesterol was measured after the adrenal tissue was dried *in vacuo* over P_2O_5 for two days and at 80° C. for two hours, and then pulverized to a fine powder. Three accurately weighed samples of the ground powder were added to chloroform, extracted in a Kahn shaker, and the Liebermann-Burchard reaction, as described by Kingsley and Schaffert (1949), applied to the dehydrated and decolorized chloroform extracts. Perfusate cholesterol was estimated by similar colorimetric assay following florosil chromatography of the chloroform extractable lipid material. Tissue ascorbic acid was determined by suspending the weighed glands in a known volume of ice-cold, 3% metaphosphoric acid and homogenizing them for three minutes in a cooled Waring blendor. The method of Stotz (1940) was applied to aliquots of this homogenate. The ascorbic acid content of adrenal effluents was determined by the 2,4-dinitrophenylhydrazine method of Roe and Kuether (1943).

CONTROL EXPERIMENTS

Initial studies indicated that suitable conditions for *in vitro* steroidogenesis were afforded if intact calf adrenals were perfused at body temperature with an oxygenated physiological saline. Glucose, in concentration of 0.2%, was added to supplement the tissue store of energy-liberating metabolites. Although it was later observed that the calcium and magnesium salts could be omitted from the artificial fluid without affecting the output of corticoids by ACTH-stimulated glands, their inclusion in the APM was continued. When oxygenation was omitted and the circulating medium gassed with nitrogen to expel air dissolved by agitation, an almost negligible production of adrenal cortical steroids was obtained. In three experiments, the mean UV, PS, and FS outputs of ACTH-stimulated glands perfused for 90 minutes with and without oxygenation of the APM were as follows: without O_2—492, 88, 420 μg.; with O_2—2100, 1115, 2020 μg.

A non-pulsatile hydrostatic pressure of 75 mm. Hg was selected after preliminary experiments had indicated that a perfusion pressure of 110 mm. increased the possibility of tears in the perfused organs without augmenting the release of corticosteroids. The obvious advantage of perfusion at body rather than at room temperature was observed in one experiment in which the costeroidogenesis of contralateral groups of adrenals differed by a factor of three. Most of the perfusion studies were conducted for two hours because it was found that the steroid output declined somewhat during the third hour.

Before initiating studies of the influence of various physiological substances upon adrenocortical activity, it was necessary to determine whether under the perfusion conditions employed, the corticoid outputs of contralateral groups of adrenals, similarly treated and simultaneously perfused in individual systems, were comparable. The mean ±S.D. of the percentage differences in the UV, PS, and FS corticoid outputs of contralateral groups similarly treated in 8 studies were 0.1 ± 5.5, 0.3 ± 4.6, and 0.6 ± 5.3, respectively.

EXPERIMENTS AND RESULTS

ACTH and steroidogenesis. All the experiments summarized in Table 1 were conducted with intact calf adrenals, mounted in a multicycle apparatus about three hours after the death of the animals, and perfused at 37.5° C. with the steroid-free, oxygenated artificial medium (APM) already described. Banks of four glands were perfused in parallel for two

TABLE 1. CORTICOID OUTPUT OF CONTRALATERAL CALF ADRENALS PERFUSED WITH AND WITHOUT ACTH ADDED TO AN ARTIFICIAL MEDIUM

ACTH conc. I.U./L.	No. of expts.	Micrograms/10 gm. adrenals/2 hr.		PS output relative to UV %
		UV (240 mμ)	PS chromogens	
0.1	2	938		
0		784		
Net*		154		
1	2	1108		
0		826		
Net		282		
5	2	1632	659	40.4
0		911	295	32.4
Net		721	364	50.5
10	5	2586	1169	45.2
0		1152	347	30.1
Net		1434	822	57.3
25	5	2976	1329	44.7
0		1313	373	28.4
Net		1663	956	57.5
50	5	2654	1236	46.6
0		1102	320	29.0
Net		1552	916	59.0
100	5	2147	1080	50.3
0		915	287	31.4
Net		1232	793	64.4

* Net output due to ACTH stimulation.

hours with ACTH[2] while the four contralateral organs were concurrently perfused without ACTH. It is evident that the adrenals perfused in the absence of added ACTH secreted significant amounts of corticoids. The secretory activity was markedly augmented by the addition of ACTH to the APM, as evidenced by the net corticoid values, calculated by subtracting the output without ACTH stimulation from that obtained with it. Glands perfused eight hours after their removal from the influence of endogenous corticotrophin still exhibited a significant adrenocortical secretory rate in the absence of added ACTH.

It was of interest to ascertain whether ACTH can induce not only a quantitative change in output but also a qualitative change in the pattern of secretion. The data of Table 1 demonstrate: (1) that the addition of ACTH increased the total output of corticoids (UV) as well as the output of the Porter-Silber chromogens (PS), (2) that the net increase in corticoid

[2] Armour's *ACTHAR*.

secretion due to corticotrophic stimulation was a direct function of the ACTH concentration from 0.1 to 25 I.U. per liter, and (3) that the corticoid production following ACTH-stimulation was composed of a higher percentage of PS chromogens than the output of unstimulated contralateral glands. Within the concentration range of from 5 to 100 I.U. ACTH per liter, the PS chromogenic fraction averaged 45.4% of the total (UV) output. In contrast, this fraction averaged only 30.3% of the total corticoid output in the 22 control perfusions without ACTH. Other perfusion stud-

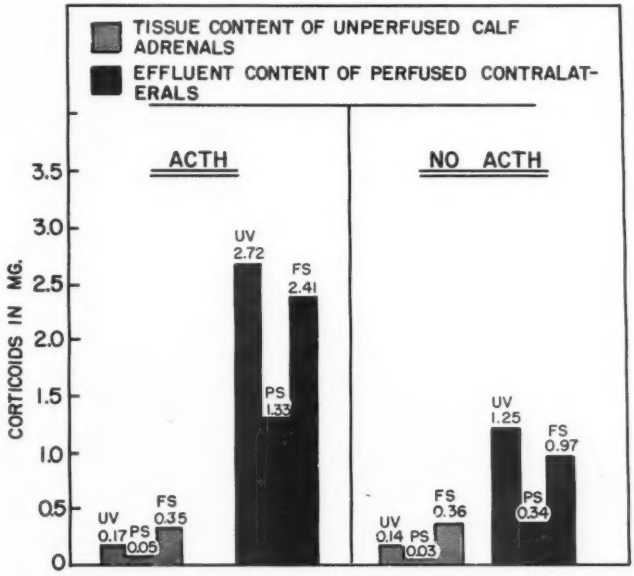

FIG. 1. The data of two experiments confirming that the isolated calf adrenal perfused in vitro, with or without ACTH added to an artificial medium, synthesizes corticoids as measured by the UV, PS, and FS methods.

ies conducted without ACTH stimulation and shown in Figure 3 (infra vide) also demonstrate that the 17-hydroxylated corticoids constituted less than 35% of the total amount released.

Figure 1 demonstrates that the corticoids found in the effluents of either ACTH-stimulated or unstimulated adrenals perfused for two hours with the artificial medium represent not merely steroidal substances already present in the glands and released during the perfusion process but an actual biosynthesis of corticoids under the simple *in vitro* conditions described. Three independent corticoid measurements (UV, PS, and FS) show that the amount secreted by four adrenals perfused with or without ACTH, was significantly greater than the tissue content of the four unperfused contralateral control glands. Evidence for the presence in the

effluents of compounds F, E, and B (the major components) as well as smaller amounts of other corticoids was established by paper chromatographic fractionation of the corticoid extracts according to methods already described in detail (Ungar, Rosenfeld, *et al.*, 1954). The zones exhibited the same blue tetrazolium and 2,4-dinitrophenylhydrazine color reactions, UV absorption, and mobility as standards chromatographed simultaneously. In four perfusates obtained following ACTH stimulation, the average amounts of F, E, and B found in the eluates from paper chromatographic zones were 1.01, 0.55, and 1.26 mg., respectively. There were approximately 0.4 mg. of unidentified compounds (X) more polar than F. The data are presented in Table 2.[3]

Figure 2 demonstrates the effect on steroidogenesis of increasing the time interval between the death of the animals and perfusion of their isolated,

TABLE 2. MILLIGRAMS OF VARIOUS CORTICOIDS IN THE EFFLUENT OF FOUR CALF ADRENALS PERFUSED FOR TWO HOURS WITH AN ARTIFICIAL MEDIUM CONTAINING ACTH*

Expt. No.	Eluted paper chromatographic zones			
	Phosphomolybdic	Porter-Silber		
	B	F	E	X†
1	0.88	0.85	0.47	0.40
2	1.22	1.29	0.83	0.57
3	1.22	1.10	0.65	0.51
4	1.72	0.80	0.26	0.28

* 25 I.U./L.hr. of added ACTH.
† Unidentified compounds more polar than F.

ACTH-stimulated adrenals. Glands, kept in citrate-saline solution at 4° C. and perfused 5 hours after the contralaterals, secreted about 35% less corticoids than the latter. The steroidal output of adrenals perfused the next day showed an even greater decline particularly in the net output due to ACTH stimulation.

Blood and APM as perfusion media. Experiments were conducted to compare the corticoid outputs of contralateral adrenals, perfused with APM and homologous whole blood. Calf blood was collected at the abattoir in a cold ACD solution, which provided a glucose level in the citrated blood of about 500 mg.%. Besides the ions from inorganic salts, the only other blood constituent present in the APM was 200 mg.% of glucose. The results from four perfusions conducted with 25 I.U./L. of ACTH indicated that the UV and PS corticoid output of the APM-perfused glands was approximately the same (<7% difference) as that of the blood-perfused contralateral organs.

Adrenal cholesterol. The dynamic *in vivo* fluctuations in adrenal cholesterol, generally believed to be related to the biosynthesis and release of the cortical hormones, prompted us to investigate the relationship of cholesterol

[3] The author is grateful to Dr. F. Ungar for his help in chromatographing the extracts.

TABLE 3. CHOLESTEROL CONCENTRATION OF CONTRALATERAL SETS OF FOUR POOLED CALF ADRENALS

Group I. Both sets unperfused			Group II. One set perfused for 4 hours with calf blood			Group III. One set perfused for 4 hours with blood contg. ACTH*		
Unperfused glands	Unperfused contralaterals	Difference	Unperfused control glands	Perfused contralaterals	Change due to perfusion without ACTH	Unperfused control glands	Perfused contralaterals	Change due to perfusion with ACTH
mg.%	mg.%	%	mg.%	mg.%	%	mg.%	mg.%	%
321	318	0.9	351	424	+20.8	399	440	+10.3
469	462	1.5	333	401	+20.4	374	435	+16.3
332	330	0.6	351	441	+25.6	372	408	+ 9.7
329	324	1.5	392	455	+16.1	355	385	+ 8.5
327	327	0.0	401	473	+17.9	381	412	+ 8.1
324	319	1.5						
423	416	1.7						
331	324	2.1						
350	346	1.1						
331	322	2.7						
415	411	1.0						
359†	355	1.3	366	439	+20.1	376	416	+10.6

* 25 I.U./L.hr. of added ACTH.
† The figures on this line represent the mean values.

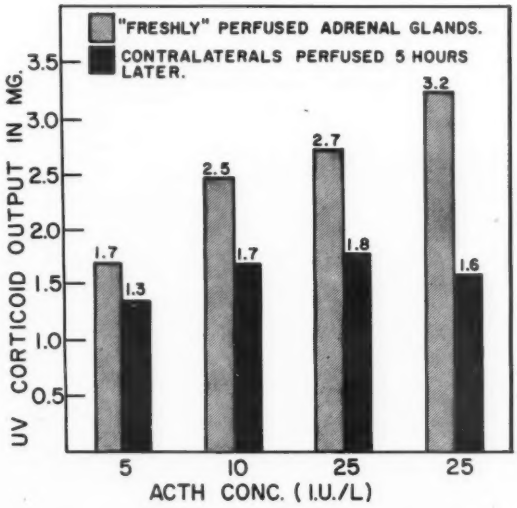

FIG. 2. Corticoid secretion in four experiments conducted at different ACTH levels. The hatched bar represents the UV output by a group of four control adrenals, perfused within 3 hours after the death of the animals, the solid bar, the UV output by the four contralateral glands, perfused 8 hours after death.

TABLE 4. CHOLESTEROL CONCENTRATION OF FOUR UNPERFUSED CALF ADRENALS AND OF THE CONTRALATERAL SETS PERFUSED FOR THREE HOURS WITH AN ARTIFICIAL MEDIUM (APM)

Group IV. No ACTH added to the APM			Group V. ACTH* added to the APM		
Unperfused control glands	Perfused contra-laterals	Change due to perfusion without ACTH	Unperfused control glands	Perfused contra-laterals	Change due to perfusion with ACTH
mg.%	mg.%	%	mg.%	mg.%	%
368	344	−6.5	396	352	−11.1
384	364	−5.5	425	357	−16.0
375	351	−6.4	343	275	−19.8
335	317	−5.4	456	348	−23.7
389	361	−7.1	392	317	−19.1
370†	347	−6.2	402	330	−17.9

* 20 I.U./L./hr. of ACTH.
† The figures on this line represent the mean values.

to adrenocortical secretion in our isolated system. Group I of Table 3 demonstrates that the cholesterol concentration of unperfused contralateral sets of four pooled calf adrenals is very similar. When one set, however, was perfused for four hours with homologous blood, its cholesterol concentration was appreciably greater than that of the unperfused contralateral set, as shown by Group II. Similar albeit smaller increments of cholesterol were observed following perfusion with blood containing added ACTH (Group III).

When unstimulated glands were perfused with the artificial medium containing only inorganic salts and glucose, there was a depletion of cholesterol (Table 4), and a concomitant "basal" secretion of corticoids.

TABLE 5. CHOLESTEROL CONCENTRATION OF CORTICAL AND MEDULLARY TISSUES POOLED FROM CONTRALATERAL SETS OF FOUR CALF ADRENALS

Cortical* and medullary† tissue from unperfused glands				Medullary tissue from unperfused controls and contralateral glands perfused for 3 hours with APM contg. ACTH		
Cortical tissue	Contra-lateral cortices	Medullary tissue	Contra-lateral medulles	Unperfused medullary tissue	Perfused medullary tissue	Change due to perfusion with ACTH
mg.%	mg.%	mg.%	mg.%	mg.%	mg.%	%
362	356	503	506	548	552	+0.7
340	334	481	488	600	596	−0.7
383	397	541	551	599	592	−1.2
360	350	494	495	540	544	+0.7
386	381	562	569	572	564	−1.4
422	402	574	563	504	500	−0.8
390	376	498	500	500	502	+0.4
				556	556	0.0
374		523		552	551	−0.2

* Includes capule.
† The medullary tissue averaged about 30% of the wet weight of the whole glands. The figures on the last line represent the mean values.

The addition of ACTH to the APM caused a three-fold increase in cholesterol depletion as well as corticoid output. If cholesterol is the natural primary precursor of the corticoids, one would expect a greater decline in the cholesterol content of ACTH-stimulated adrenals in view of their significantly greater output of corticoids. Inasmuch as analyses of the perfusates revealed small amounts of cholesterol-like substances insufficient to account for the glandular losses, it appears that the cholesterol depleted from the adrenals had not been merely released into the circulating medium but had been metabolized by the glands.

It remained to be demonstrated whether the observed depletion was incurred by the cortex, the medulla, or both. Table 5 shows that the medullary tissue, which constitutes about 30% of the total weight of fresh calf adrenals, excised from animals weighing around 225 pounds, has a much higher cholesterol concentration than the corresponding cortical tissue. In fact, the total cholesterol content of the medulla approximates that of the cortex if one considers their relative weights and cholesterol concentrations. Despite its high content, however, there was no appreciable loss of cholesterol from the medullary portion of glands perfused for three hours with concentrations of ACTH varying from 10 to 100 I.U./L./hr. Thus, the loss in cholesterol exhibited by the perfused, intact adrenal (Table 4) can be attributed to a selective or specific depletion of the organ's cortical tissue cholesterol.[4]

Ascorbic acid. In view of the high ascorbic acid concentration in the adrenal and the wide fluctuations it exhibits under conditions of increased secretory activity, it was of interest to investigate the influence of this vitamin on the adrenocortical function of isolated glands. Preliminary studies revealed that the ascorbic acid concentrations of unperfused contralateral groups of four pooled adrenals were comparable (% difference $<3\%$). Group I of Table 6 demonstrates that perfusion of calf adrenals with homologous whole blood for one hour reduces their ascorbic acid content to a very low level. After perfusion for two hours, no ascorbic acid could be detected in the glands either chemically or histochemically ($AgNO_3$ staining). Group II indicates that a similar drastic depletion occurs even if the adrenals are perfused with blood containing 100 mg.% of ascorbic acid, added alone or with 400 mg.% of glutathione (sodium salt). To be sure, the perfusion conditions do not duplicate *in vivo* ones, and their artificiality probably accounts for some of the loss of this unstable vitamin. Mere incubation of glands in blood at 37.5° C. induced about a 50% diminution in their ascorbic acid content after two hours.

The possibility remained that a large portion of the glandular ascorbate content was "washed out" during the perfusion process. To explore further the striking depletion in perfused adrenals, one set of unperfused organs

[4] The medulary tissue was preferably analyzed because of the feasibility of obtaining practically all of it uncontaminated with other tissue.

was analyzed directly and found to have a total content of 22.4 mg. The contralateral set, kept in citrate-saline at 3° C. was perfused 20 minutes later with a liter of APM containing 25 I.U. ACTH (conditions adequate for stimulation of respiratory and steroidogenic functions), while a control liter of APM containing 22.4 mg. of ascorbic acid was concurrently recycled in another perfusion system under the same conditions but without mounted organs. At the end of two hours, there was practically no vitamin C in the adrenal effluents (or glands), whereas more than 70% of the added vitamin was recovered from the control perfusion medium. A second ex-

TABLE 6. ASCORBIC ACID CONCENTRATION OF SETS OF FOUR UNPERFUSED CALF ADRENALS AND OF THE CONTRALATERAL GLANDS PERFUSED FOR ONE HOUR

Group I. Perfusion with whole blood			Group II. Perfusion with blood containing 100 mg.% ascorbic acid		
Unperfused control glands	Perfused contra-laterals	Change due to perfusion	Unperfused control glands	Perfused contra-laterals	Change due to perfusion
mg.%	mg.%	%	mg.%	mg.%	%
158	19	−88	136	13	− 90
146	13	−91	160	3	− 98
132	16	−88	128	0	−100
184	7	−96	135	0*	−100
122	9	−93			
148	13	−91	140	4	− 97

* Perfused with 100 mg.% ascorbic acid and 400 mg.% glutathione.
The figures on the last line represent the mean values.

periment corroborated these results. In contrast to the ACTH-stimulated adrenal effluents, an average of 25% (3 experiments) of the original glandular ascorbic acid was recovered in the perfusates of unstimulated adrenals. Thus, it appears that while some of vitamin content of perfused, ACTH-stimulated adrenals is probably destroyed and released as a result of the physical conditions of perfusion, a considerable portion is actually "utilized" or metabolized by the actively respiring and secreting glands.

The aforementioned rapid exhaustion of the ascorbic acid content of perfused adrenals provided an experimental tool for investigating corticoid biogenesis in glands essentially devoid of the vitamin. Group I of Table 7 represents two sets of four calf adrenals, simultaneously perfused for three hours with homologous whole blood, one without ACTH and the contralateral set with 1.4 U.S.P. units/L./hr. of ACTH.[5] They were provided each hour with fresh changes of liver-perfused blood containing no detectable amounts of ascorbic acid. Corticoid output was calculated by subtracting the blood values before adrenal perfusion from those found in the adrenal effluents. It is evident that both the unstimulated and stimulated glands elaborated appreciable quantities of corticoids during the

[5] Upjohn's *Corticotrophin.*

third hour of perfusion despite the relative absence of vitamin C in the system at this time.

Another set of four calf adrenals (Group II) was perfused for six hours with fresh hourly changes of blood containing no demonstrable vitamin C. After three hours without corticotropic stimulation, the glands were perfused for an additional three hours with 1.4 U.S.P. units/L./hr. of ACTH. The addition of ACTH induced an intense activation of steroidogenesis in spite of the practically zero level of ascorbic acid in the blood and glands, and resulted in a secretion of over 5 mg. of corticoids during

TABLE 7. HOURLY CORTICOID OUTPUT OF FOUR CALF ADRENALS PERFUSED WITH OR WITHOUT ACTH ADDED TO FRESH CHANGES OF HOMOLOGOUS BLOOD

Perfusion period	I. Secretion with and without ACTH (2 sets of glands)		II. Secretion before and after ACTH (same set of glands)	
	UV micrograms per hour	PS micrograms per hour	UV micrograms per hour	PS micrograms per hour
Unstimulated glands				
First hour	—	200	1472	920
Second hour	1300	250	1142	530
Third hour	1400	650	668	50
*Stimulated glands**				
First hour	880	800	3078	500
Second hour	1590	1350	1404	—
Third hour	1380	750	549	—

* In the case of Group II, the first, second, and third hours of stimulation represent the fourth, fifth, and sixth hours of continuous perfusion.

The analyses were done by Dr. F. Ungar.

the last three hours of a 6-hour perfusion. These studies strongly suggest that the normally occurring high levels of vitamin C in the adrenal are not essential for the biogenesis of cortical hormones or the biosynthetic activation induced by ACTH, and that steroidogenesis and secretion can occur in glands relatively free of the vitamin and receiving little or none via the bloodstream.

A series of 17 perfusion studies was conducted to study steroidogenesis in both unstimulated and ACTH-stimulated glands perfused with the APM containing 100 mg.% of either ascorbic acid, glutathione, or both (Na salts). These substances did not exert a significant effect on the corticoid secretory rate either individually or together, and, when added either separately or together to suboptimal amounts of ACTH, did not potentiate or antagonize the stimulatory effect of the corticotrophic hormone. Glutathione (in its reduced form) was included in these studies in view of its high content in the normal adrenal and its putative relationship to the stabilization and action of ascorbic acid in tissues.

Glucose and amino acids. In view of the authenticated homeostatic role of the adrenal cortex in protein and carbohydrate metabolism, as manifested by its participation in gluconeogenesis, glycogen deposition, carbo-

hydrate utilization, etc., it was desirable to investigate the response of the cortex to widely different levels of glucose and amino acids. Groups of glands were perfused with APM containing varying amounts of glucose up to 600 mg.% whereas the contralateral control groups were perfused with a level of 60 mg.%. Contralateral groups were also perfused with APM containing 50 and 0 mg.% amino acid nitrogen (provided by a casein hydrolysate supplemented with dl-tryptophane). The normal range of these nutrients in bovine blood is 40–60 and 4–8.5 mg.%, respectively. The elevated levels employed neither stimulated nor inhibited the release of adrenal cortical hormones from isolated, perfused calf adrenals.

Na/K imbalance. To ascertain whether the cortex can adapt its secretory rate autonomously to alterations in electrolyte concentrations, a set of adrenals was perfused with a modified APM, containing 125 mEq./L. of Na and 25 mEq./L. of K, whereas the APM of the contralateral control organs contained 155 and 3.8 m. eq./L. of Na and K, respectively. The control Na/K ratio of 40 resembles the normal proportion of these plasma ions as opposed to the experimental ratio of 5, which is abnormally low and simulates the deficiency of Na and excess of K that obtain in adrenalectomy or adrenal cortical insufficiency. In the two experiments conducted, this appreciable Na/K imbalance did not alter the secretory activity, as measured by the UV, PS, and FS corticoid outputs.

Adenosine triphosphate (ATP). The disparity between the low content of stored corticoids in the calf adrenal (Fig. 1) and its high secretory rate when perfused suggests an active adrenocortical biosynthetic capacity. The intimate role of ATP in cellular synthetic and energy-liberating processes prompted us to investigate the effect of added ATP on the production of cortical hormones by isolated, unstimulated and ACTH-stimulated adrenals. Groups of glands were perfused with a liter of APM to which 125 mg. of ATP (Na salt) were added every 30 minutes and the control groups with an equivalent amount of adenosine monophosphate (AMP). In three experiments without and three with ACTH stimulation (10 I.U./L.), ATP enhanced the total adrenocortical output (UV) an average of 21% and 32%, respectively. The production of Porter-Silber chromogens exhibited comparable increments following perfusion with this organic phosphate.

Hormonal substances. The possibility of an acute, direct conditioning action of epinephrine, histamine, thyroid substances, and gonadotrophin upon adrenocortical secretory activity was explored. Sets of glands were perfused with APM containing 100 μg.% adrenalin chloride, 400 μg.% histamine phosphate, or 200 μg.% of organic iodine as thyroglobulin[6] and thyroxine while the contralateral groups were simultaneously perfused

[6] Although thyroglobulin does not normally appear in the blood stream, it was employed because it stimulates oxygen utilization *in vitro* while thyroxine does not.

without these added constituents. Their normal plasma values are approximately 0.1 μg.%, 10–20 μg.%, and 10 μg.% or less, respectively. Chorionic gonadotrophin[7] (5000 I.U./L.) was perfused through groups of adrenals excised from 2-month old animals to ascertin the effect of this hormone on steroidogenesis. The results, graphically illustrated in Figure 3, indicate that very high levels of histamine, thyroid substances, and chorionic gonadotrophin exerted neither an acute stimulatory nor suppressive action on adrenocortical activity. Production was slightly decreased by adrenalin but this could have been due to a vasoconstrictory effect with consequent lowering of the flow rates through the glands.

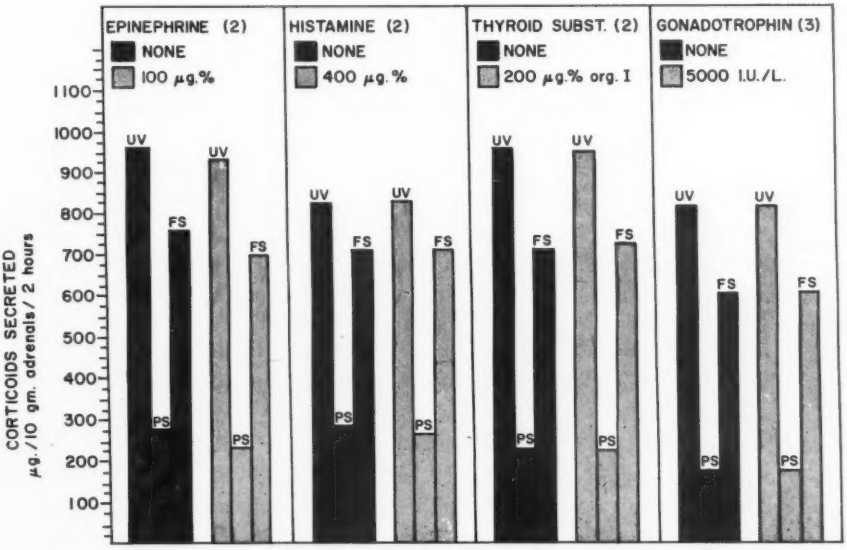

FIG. 3. Mean corticoid out as estimated by the UV, PS, and FS methods, secreted by contralateral groups of calf adrenals, perfused with and without the above hormonal substances added to the artificial medium (APM). The figures in parentheses designate the number of experiments.

DISCUSSION

The findings that the perfused calf adrenal spontaneously secretes corticosteroids in the absence of added ACTH and that there is an appreciable depletion of its ascorbic acid and cholesterol contents incident to perfusion is at variance with those of Hechter (1951). The present results differ from those of Hechter and his coworkers in two other respects. In studies with media other than whole blood containing as much as 30 I.U./L. of ACTH, Macchi and Hechter (1954a) found little or no steroidogenesis when bovine adrenals were perfused without red blood cells. When

[7] Parke, Davis *Antuitrin "S."*

whole blood was employed, the same workers (1954b) observed that increasing concentrations above 0.001 I.U./L. augmented the magnitude of response, which was maximal at 0.1 I.U./L. In the present studies, 100-fold the latter concentration was needed in the case of calf adrenals perfused with an artificial medium. The reason for this difference in ACTH requirements is not apparent at this time. In view of the comparable corticoid outputs by contralateral glands perfused with whole blood and artificial media, it would appear that although red cells are not needed for maximal stimulation of the glands, their presence lowers the concentration of ACTH required.

Under the present perfusion conditions, the calf adrenal is an active preparation, capable of steroidogenesis and exhibiting variable degrees of responsivity depending upon the concentration of its tropic hormone (ACTH) used for perfusion. Marked fluctuations in the titre of other physiological substances normally occurring in blood, viz., ascorbic acid, glutathione, glucose, amino acids, potassium and sodium, adrenalin, histamine, gonadotrophin and thyroid substances, failed to elicit a similar direct and acute adrenocortical response when added to the artificial perfusion medium. In fact, perfusion of ACTH-stimulated adrenals with homologous whole blood (rather than with APM) resulted in no apparent increase in steroidogenic activity despite its galaxy of nutritive and other constituents. Dugal and Thérien (1952) recently claimed that ascorbic acid potentiates the action of ACTH on the adrenal cortex. Moreover, the administration of large parenteral doses of this vitamin has been reported to increase the fall in adrenal cholesterol and to have other ACTH-like effects (Dugal, 1951). The perfusion studies with ascorbic acid and glutathione, added separately or jointly, did not exhibit a direct excitatory effect nor did they potentiate the action of suboptimal amounts of ACTH. It must be emphasized, however, that the short-term studies reported herein were designed to ascertain factors regulating or modifying the acute response of the adrenal cortex, such as occurs in the "alarm reaction." Chronically, the cortex, like any other tissue, would depend on and accordingly be influenced by a host of nutritive and other substances in the circulation.

Of all the substances tested, other than ACTH, which may be found in the blood stream, adenosine triphosphate (ATP) was the only one which augmented the steroidogenesis of isolated glands. Its acceleratory effect was observed both in the absence and presence of added corticotrophin. To be sure, the high levels employed in the perfusation fluid were unphysiological, and normally, the cortex probably synthesizes its own ATP content. Recently, Grenier and coworkers (1952) injected ATP intravenously and observed an eosinopenia and increased 17-ketosteroid excretion. They postulated that ATP may exert a direct effect on the pituitary-adrenal mechanism. The results reported herein suggest that

an elevated blood titre following i.v. administration may act as a direct excitant of the adrenocortical secretory mechanism independent of ACTH release. At present, it cannot be stated whether ATP acted directly in the biosynthesis of the corticoids or indirectly as, e.g., in the reactions which provide energy for the syntheses and other metabolic activities of the gland. The vital role ATP plays in phosphorylation processes and the demonstration by Reiss (1950) that ACTH accelerates phosphorylation lends support to the view of its direct participation in costeroidogenesis. Moreover, it has recently been shown with adrenal homogenates that TPN, a co-factor for the 11-β hydroxylation system, can be replaced by a combination of ATP and DPN (Hayano and Dorfman, 1953).

That adrenocortical secretion, although decisively enhanced by ACTH, was not wholly dependent upon it but occurred at a "basal" rate without its addition to the APM, conforms to the generally accepted biological concept that hormones do not create or initiate new physiological processes but merely act catalytically to alter the rate of intrinsic ones. The data presented agree with those of Vogt (1951), who observed a steady rate of secretion from dog adrenals perfused without ACTH, in contradistinction to those of Hechter (1951), who found either a slight or no release of FS corticoids from unstimulated, perfused bovine adrenals. It has already been mentioned that glands, kept for eight hours after their removal and rendered essentially devoid of blood by preliminary perfusion with citrate-saline, exhibited appreciable steroidogenic activity when perfused with an artificial medium containing no ACTH. In view of this 8-hour time lapse as well as the rapid inactivation of ACTH *in vitro* by adrenal and other tissues even at 6° C. (Geschwind and Li, 1952), it is probable that very little, if any, active endogenous ACTH remained in the isolated glands at the time of mounting.

The observation that ACTH "favors" the secretion of Porter-Silber chromogens indicates that ACTH not only stimulates steroidogenesis but induces a qualitative change in the pattern of biogenesis and secretion. The Porter-Silber chromogenic fraction represents 17,21-dihydroxy-20-ketosteroids rather selectively (Porter and Silber, 1950)—that is, 17-hydroxycorticosteroids, as exemplified by compounds F and E—in contrast to the UV fraction which represents all corticoids with an alpha-beta unsaturated ketonic grouping in ring A and includes Compounds F and E, as well as B and A, which are not 17-hydroxylated. It is possible that the discharge of ACTH *in vivo* not only augments the adrenocortical secretory rate to meet the increased demands for corticoids imposed by the stress conditions provoking its release, but also alters the spectrum of steroidal secretion, thereby providing the organism with a greater proportion of 17-hydroxylated corticoids. There is much evidence to support the view that the latter are more potent in the homeostatic adaptation of the stressed animal to both internal and external environmental changes.

Considerable data on the cholesterol and ascorbic acid concentrations of calf adrenals have been presented. It should be emphasized that these values do not necessarily reflect their concentrations in normal glands. In every case, the adrenals were obtained from animals transported to a slaughterhouse and herded closely together until elevated by a hind leg and decapitated. Their history as well as the complete absence of eosinophils in their blood at the time of slaughtering strongly suggest that the animals had experienced considerable stress before death. Consequently, the adrenals employed were probably depleted in varying degrees with respect to both their cholesterol and ascorbic acid contents. The variable nutritional regime, age, weight, and strain of the animals would also be conducive to intial concentration differences. The studies did reveal, however, that there is a loss of cholesterol from isolated calf adrenals, perfused in the absence of ACTH, and that this loss is greater, as is the corticoid output, when the glands are stimulated with ACTH. From both whole animal experiments (Conn *et al.*, 1950) and isolated bovine adrenal perfusions (Hechter, 1951), it has been reported that blood cholesterol may be utilized as a precursor by the adrenal glands in their synthesis of corticosteroids. Perfusion with an artificial, cholesterol-free medium demonstrated that appreciable steroidogenesis can occur in the absence of a circulating source of cholesterol and, hence, is not dependent upon the transformation of blood cholesterol.

In view of the failure to maintain or replace the ascorbic acid content of adrenals perfused with high levels of the vitamin (and glutathione), it is possible that the adrenal of the calf (an animal which does not require an exogenous source of the vitamin), synthesizes its own ascorbic acid rather than obtaining it via the circulation. Ascorbic acid studies with depleted glands demonstrated that corticogenesis and secretion occurred *in vitro* in adrenals essentially devoid of the vitamin and receiving none via the circulation. They suggest that the specific participation of ascorbic acid is the series of events following ACTH stimulation in the whole animal may not be obligatory. The observation that adrenocortical function is maintained in experimental animals and in patients with advanced scurvy does not preclude the possibility of its direct participation in steroidogenesis inasmuch as the small amounts which remain may be sufficient to support the process. The presence of high concentrations as normally occur, however, would not appear to be essential, since in our isolated system, biosynthesis and secretion occurred in the absence of detectable amounts. The evidence does not exclude an indirect role in adrenocortical activity by virtue of its participation in cellular respiratory and other metabolic activities. Perhaps, this role can be fulfilled by other reducing substances, i.e., glutathione, so that adrenocortical function would be maintained in its virtual absence.

Recently, Hughes and Coworkers (1952) presented data to support their

hypothesis that vitamin C is essential to the formation of the oxy-type of adrenal cortical hormones like hydrocortisone and cortisone as opposed to desoxycorticosterone. It has also been reported that vitamin C is capable of serving as a coenzyme in a biological oxidation system (Sealock and Goodland, 1951) and that desoxycorticosterone can be oxidized *in vitro* to cortisone by adrenal cortical tissue and added ascorbic acid (Seneca *et al.*, 1950). Purification and fractionation of perfusate extracts (Table 7, Group II) by means of the paper chromatographic technique of Zaffaroni (1950), and subsequent isolation and identification revealed that glands with no detectable vitamin C content had biosynthesized appreciable amounts of hydrocortisone and corticosterone, both 11-oxysteroids.

SUMMARY AND CONCLUSIONS

The results of 134 adrenal perfusion studies are presented. Steroidogenesis was investigated under relatively simple and defined experimental conditions by perfusing intact calf adrenal glands *in vitro* with an artificial medium (APM) containing balanced inorganic salts and glucose. The corticoids in the effluents were assayed by ultraviolet absorption at 240 mμ, the Porter-Silber reaction, and the Hollander procedure for formaldehydogenic steroids. An appreciable biogenesis and secretion occurred when the glands were perfused at body temperature with the artificial medium well-oxygenated. The production of corticoids was negligible in the absence of oxygen. ACTH added to the oxygenated perfusation fluid not only induced a marked, dose-dependent augmentation of output but also a qualitative change in the pattern of secretion, as evidenced by the increase in the 17-hydroxylated (PS) fraction of the total corticoids secreted. The corticoid outputs of ACTH-stimulated, contralateral groups perfused with APM and homologous whole blood were comparable.

The depletion of cholesterol, incident to perfusion with APM alone and incurred selectively by the cortical tissue, was enhanced three-fold by the addition of ACTH and paralleled the corticoid output with and without corticotrophic stimulation. A similar but more drastic depletion of the ascorbic acid content followed perfusion of isolated glands. It was demonstrated, however, that steroidogenesis and secretion can occur *in vitro* in adrenals essentially devoid of the vitamin and receiving none via the circulation. Evidence was adduced to indicate that the depletion in the cholesterol and ascorbic acid contents of isolated organs is not merely a result of the physical conditions of perfusion but that a considerable portion is actually "utilized" or metabolized by the actively secreting organs.

Markedly elevated levels of ascorbic acid, glutathione or both perfused through unstimulated or ACTH-stimulated glands did not reveal any acute, direct effect on adrenocortical activity, nor did they potentiate or antagonize the stimulatory effect of ACTH. High levels of other physiological constituents normally occurring in blood, viz., glucose, amino

acids, potassium (with a low sodium level), adrenalin, histamine, chorionic gonadotrophin, and thyroid substances also failed to induce either an acute excitation or inhibition of the secretory rate. The only physiological substance, other than ACTH, which exhibited a direct and acute, stimulatory action on steroidogenesis was adenosine triphosphate.

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THE DURATION OF STIMULATION OF THE FOWL'S ANTERIOR PITUITARY FOR PROGESTERONE-INDUCED LH RELEASE[1]

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INVESTIGATIONS on the stimulation of the hypophysis of rabbits and rats have shown that in mammals species difference exists in regard to the duration of stimulation necessary to cause ovulation (Sawyer, Markee, and Hollinshead, 1947; Everett, 1952; and Everett and Saywer, 1953). Van Tienhoven *et al.* (1954) suggested in a recent study that in the hen stimulation of the anterior pituitary gland and release of LH from that gland might take place concurrently.

In order to investigate this hypothesis further, progesterone was given on the day of lay of the terminal egg of a clutch, thus inducing premature ovulation of the first follicle of the next clutch (Fraps and Dury, 1943). This technique was very useful for controlling the time when stimulation started, because of the variation in the time of normal ovulation that existed in the groups of hens used in these experiments.

Atropine (Zarrow and Bastian, 1953) and Dibenamine (van Tienhoven *et al.*, 1954), are effective agents in blocking the "spontaneous" and the progesterone-induced ovulation in the hen. These agents were used in a series of experiments to determine the duration of stimulation of the fowl's hypophysis.

MATERIALS AND METHODS

Single Comb White Leghorns of ages between 9 months and 2 years and White Plymouth Rock hens of 6–9 months of age were kept in individual laying cages. Only hens were used which laid 3 or more eggs per clutch and which showed breaks between clutches of not more than one day. The birds received an 18% protein all-mash ration and water *ad libitum*. Three electric 75-watt light bulbs provided artificial light from 5 A.M. to 7 P.M. throughout the experiment.

The birds were palpated every morning between 7 and 8 A.M. and again at 9 P.M. on the day of injection. The interpretation of the palpation results were the same as previously (van Tienhoven *et al.* 1954). Oviposition was checked every hour from 7 A.M. till 5 P.M. and again at 9 P.M. Some birds were used more than once in an experiment, but each time a bird had gone through at least 2 normal laying cycles before she was used again.

In the Dibenamine experiment progesterone was dissolved at a concentration of 1

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mg./cc. in a mixture of equal parts of ethanol and propylene-glycol. Three parts of this solution were mixed with two parts of physiological saline just before injection. In the atropine experiments a concentration of 2 mg./cc. was used and 1 cc. was given per bird.

Dibenamine was dissolved at a concentration of 50 mg./cc. in a mixture of 49.8 parts of propyleneglycol, 50 parts of ethanol, and 0.2 parts of HCl. Three parts were mixed with two parts of physiological saline just prior to injection.

The mixture of propyleneglycol, ethanol, and HCl in the above mentioned proportions was designated as carrier; it was mixed with physiological saline in ratios similar to the Dibenamine and progesterone solutions.

Atropine was dissolved in physiological saline at a concentration of 70 mg./cc. The subcutaneous injections were made in 2 equal portions in the skinfold between breast and thigh. For "simultaneous" injection of atropine and progesterone the atropine was injected 15 minutes prior to the intravenous progesterone injection. At the time of progesterone injection in this group the effects of the atropine, like dropping of feathers, panting, and muscular weakness were evident.

Rooster pituitaries were collected from heads which had been frozen immediately after killing of the birds. The pituitaries were crushed between glass plates and dried under vacuum. The powder was suspended in water at a concentration of 1 mg./ml. or 0.25 mg./ml. and kept frozen between experiments.

All progesterone injections were made between 2 and 3 P.M. on the day of lay of the last egg of the clutch, so that the endpoint of hypophyseal stimulation was the ovulation of the first follicle of a new clutch. Prematurity of ovulation was judged by digital palpation, using the same interpretations as van Tienhoven *et al.* (1954).

The birds to be used in an experiment were assigned at random to the treatments for each experiment.

In the atropine experiments all birds failing to ovulate prematurely were autopsied or laparotomized the next morning and the incidence of atresia recorded.

The following criteria were used for complete, partial or failure of blockade. Complete blockade was indicated by the failure to ovulate, without atresia of any of the ovarian follicles. Follicles which were atretic were designated as being due to partial blockade, while failure of blockade resulted in a plumped egg in the oviduct the morning after treatment.

EXPERIMENTS AND RESULTS

Three series of experiments were carried out in which laying hens received 0.6 mg./kg. progesterone, 0.6 mg./kg. progesterone simultaneously with 30 mg./kg. Dibenamine, or 0.6 mg./kg. progesterone followed at a 1, 3, 5, or 6 hour interval by 30 mg./kg. Dibenamine.

Data reported previously by van Tienhoven *et al.* (1954) for progesterone given alone (14 birds) and progesterone and Dibenamine given simultaneously (14 birds), were pooled with the respective data obtained in the present series of experiments because no statistically significant differences existed ($P > 0.10$).

A similar series of experiments was carried out in which atropine was used. It was found that at a dose of 350 mg./kg. in 8 out of 9 cases the premature ovulation was blocked. However, in a subsequent experiment the mortality was about 70%. At a level of 280 mg./kg., which was finally selected, the per cent blocked premature ovulations was 85 and mortality was about 5%. The results of both the Dibenamine and atropine (280 mg./kg.) data are reported in Table 1.

The Dibenamine data are not suitable for an analysis of the exact duration of hypophyseal stimulation, because of atresia, which occurred in about 20% of the cases of simultaneous injection of progesterone and Dibenamine (Everett and Sawyer, 1953). The same phenomenon was observed in the simultaneous administration of Dibenzyline 30 mg./kg., which was shown to be more effective than Dibenamine in blocking the vasopressor response of epinephrine in chickens (Harvey *et al.*, 1954). In the Dibenamine treated birds a significant ($P < 0.05$) higher incidence of blocked premature ovulations occurred in the 5-hour groups, and a highly significant ($P < 0.01$) difference existed for the zero, 1-hour and 3-hour

TABLE 1. INCIDENCE OF BLOCKADE OF PREMATURE PROGESTERONE-INDUCED OVULATIONS AT DIFFERENT TIME INTERVALS BETWEEN PROGESTERONE AND DIBENAMINE OR ATROPINE ADMINISTRATION

Interval progesterone Dibenamine	Birds inj.	Birds blocked premature ovulations		Interval progesterone atropine	Birds inj.	Birds blocked premature ovulation	
h.	*no.*	*no.*	%	*h.*	*no.*	*no.*	%
0	26	22†	84.6	0	20	17†	85.0
1	15	13†	86.7	1	12	8†	67.7
1.5	—	—	—	1.5	12	9†	75.0
2	—	—	—	2	12	6†	50.0
2.5	—	—	—	2.5	12	4	33.3
3	15	9†	60.0	3	13	0	—
4	—	—	—	4	12	0	—
5	14	7*	50.0	5	11	2	18.2
6	14	5	35.7	6	11	2	18.2
Prog. alone	19	3	15.8	Prog. alone	21	2	9.5

* Difference with progesterone alone significant at the 5% level of probability.
† Difference with progesterone alone significant at the 1% level of probability.

group when these groups were compared with the progesterone-treated controls. When the birds received atropine no significant blocking response was obtained beyond the 2-hour interval. This suggests that the Dibenamine-blocked stimulus lasts longer than the one blocked by atropine.

Because of the 100% failure of blockade in the atropine experiments at the 3 and 4-hour intervals and the non-significant difference between the 5 and 6-hour points and the controls, only the time interval between 0 and 3 hours was considered further.

In Table 2 the observations made on laparotomy of all non-prematurely ovulating birds are reported. The birds ovulating at the normal time were classified as "completely blocked" for the following reasons:

1. The normal ovulation is probably due to the low concentration of atropine at the time of normal stimulation (about 10 P.M.). This contention is supported by certain observations in the zero-hour group. Normal ovulations were entirely absent in those birds that failed to ovulate prematurely after a dose of 350 mg./kg. atropine, but did occur in 35% of the birds when the dosage was decreased to 280 mg./kg. At the 210 mg./kg. dose the frequency further increased to 50%.

2. Experiments showed that a partially stimulated follicle will not ovulate normally when an exogenous dose of gonadotrophin is given, in order to overcome the atropine

TABLE 2. INCIDENCE OF PREMATURE OVULATIONS, NORMAL OVULATIONS, BLOCKED OVULATIONS AND OF ATRESIA AT DIFFERENT TIME INTERVALS BETWEEN ATROPINE AND PROGESTERONE ADMINISTRATION

Time interval	Birds inj.	Premature ovulations		Normal ovulations (a)		Failed to ovulate (b)		Atresia (c)		Completely* blocked premature ovulations (a+b)−c	
h.	*no.*	*no.*	*%*	*no.*	*%*	*no.*	*%*	*no.*	*%*	*no.*	*%*
0	20	3	15.0	6	30.0	11	55.0	1	5.0	16	80.0
1	12	4	33.3	0	—	8	67.7	4	33.3	4	33.3
1.5	12	3	25.0	2	16.7	7	58.3	5	41.6	4	33.3
2	12	6	50.0	0	—	6	50.0	3	25.0	3	25.0
2.5	12	8	66.7	1	8.3	3	25.0	1	18.3	3	25.0
3	13	13	100.0	0	—	0	—	–	—	—	—
Prog. alone	21	19	90.5	2	9.5	0	—	–	—	—	—

* See text.

blockade of the normal stimulus. Table 3 illustrates this point. Partial stimulation of the follicles was obtained by giving progesterone and atropine at a 1½-hour interval. At 9–10 P.M., the time of "spontaneous" release of gonadotrophin, an intravenous injection of .1 mg. of rooster AP powder was made. This should lead to ovulation of the non-stimulated follicles and, if partially stimulated follicles could be ovulated, to normal ovulation of the latter.

In 4 cases no egg could be palpated the morning after injection, while in 3 of these birds a disintegrated yolk was found in the oviduct without albumen. Apparently, the breakdown of the vitelline membrane, which takes place in atresia (Rothchild and Fraps, 1945), had progressed to such an extent that at the time of release from the follicle no normal yolk was ovulated. In the progesterone-atropine group all eggs were completely normal in appearance. The table also shows that atropine itself did not cause these abnormal eggs. This leads to the conclusion that, although an apparently larger than normal dose of gonadotrophin can cause release of the partially stimulated egg from the follicle, it will not result in normal ovulation.

3. The administration of atropine alone at times corresponding to those of atropine injection in the 1.5 and 2.5-hour interval groups did not cause atresia. Out of 17 birds injected with 280 mg./kg. atropine between 4–5 P.M., 5 birds failed to ovulate, 11 ovulated at the normally expected time and 1 ovulated prematurely. No evidence of atresia

TABLE 3. EFFECT OF EXOGENOUS GONADOTROPHIN ON OVULATION OF PARTIALLY STIMULATED AND NON-STIMULATED FOLLICLES

Treatment	No. inj.	Premature ovulation	Normal ovulation	No. ovulations	Atresia	No Atresia
Prog. and atropine 1.5 hours later	12	3	2	7	5	2
Prog. and atropine 1.5 hours later and AP powder 9–10 P.M.	12	4	4	4	3*	1
AP powder and atropine at 3 P.M.	6	5	–	1	–	1
AP powder and saline at 3 P.M.	13	12	–	1	–	1

* See text.

was found. From this the conclusion was drawn that no interruption of the normal stimulation occurred, but that in 5 cases the stimulus was completely blocked.

Everett and Sawyer (1953) established that in the rat a threshold level of atropine has an all or none effect in the blockade of ovulation. This was confirmed for the hen. At a dose of atropine of 210 mg./kg. in the zero-hour group, 9 out of 15 hens ovulated prematurely, 3 ovulated normally and 3 hens failed to ovulate, while no atresia was found in any of the hens failing to ovulate prematurely.

The single case of atresia on simultaneous atropine-progesterone administration was in all probability due to an interruption of spontaneous ovulation. This is supported statistically by the highly significant difference in atresia in the zero-hour group when compared to the other groups

TABLE 4. INCIDENCE OF GONADOTROPHIN-INDUCED PREMATURE OVULATIONS IN PRESENCE OF DIBENAMINE OR ATROPINE

Treatment	Birds inj.	Birds ovulating prematurely		Birds failing to ovulate	
	no.	*no.*	%	*no.*	%
0.1 mg. AP powder+saline (s.c.)	13	12	92	1	8
0.1 mg. AP powder+carrier (i.v.)	16	13	81	3	19
0.1 mg. AP powder+30 mg./kg. dibenamine (i.v.)	14	13	93	1	7
0.1 mg. AP powder+350 mg./kg. atropine (s.c.)	6	5	83	1	17

$\chi^2 = .174$ $P > 0.10$

($P < 0.01$). It was also found that in untreated control birds about 1–3% ovulated at approximately 10 P.M. In these cases stimulation must have taken place between 2 and 4 P.M. (Rothchild and Fraps, 1949).

Data reported in Table 4 show that neither atropine nor Dibenamine interfered with the ovulatory process *per se*. It is interesting to note that, although the oviduct of the chicken has an autonomous nerve supply (Mauger, 1941), neither atropine nor Dibenamine affected the activity of the oviduct (Warren and Scott, 1935) to such an extent that internal ovulations occurred.

Huston and Nalbandov (1953) found no evidence of atresia for the first 20 days after a thread, which blocked ovulation, was introduced into the oviduct. This means that the failure to ovulate at the normally expected time does not *per se* cause atresia. Fraps and Dury (1942) showed that a submaximal dose of exogenous LH caused atresia of the hen's ovarian follicles. The results of the experiments reported in this paper and the reports by Fraps and Dury (1942), Everett (1952), and Everett and Sawyer (1953) indicated the correctness of the assumption that atresia in the progesterone-atropine experiments was due to partial stimulation of the anterior pituitary gland, by interruption of the progesterone-induced stimulus.

The regression of per cent blocked premature ovulations on time interval between progesterone and atropine administration is linear, and highly significant ($P < 0.01$). The regression coefficient was found to be -22.75 ± 4.25. The regression line is represented graphically in Figure 1. On this line a 10% increase in failure to ovulate corresponds to a time interval of 26.4 minutes between progesterone and atropine injection.

The same procedure as outlined by Everett (1952) and Everett and Sawyer (1953) for the rat was now applied to the data obtained in the hen. The interval groups between zero-hour and 3 hours were considered. A

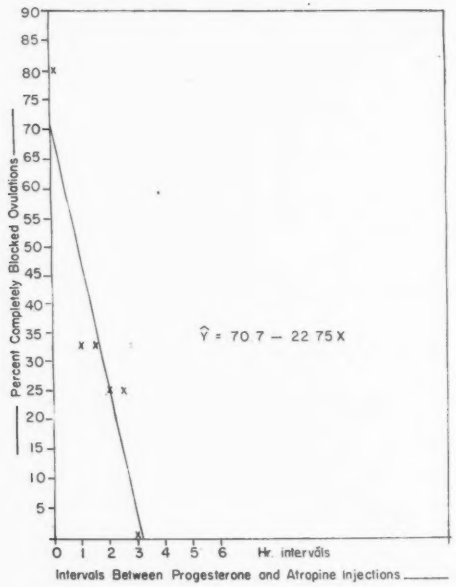

FIG. 1. Relationship between incidence of blocked ovulations and the time interval between progesterone and atropine injection.

total of 61 birds were used, while in 13 of these the stimulation was apparently interrupted. By using Hald's table XI and entering $x = 13$ and $n - x = 61 - 13 = 48$ at the levels of probability of 97.5 and 99.5%, the lower limits were found to be 11.9% and 9.7%. These values correspond to a time interval of 31.4 and 25.6 minutes respectively.

DISCUSSION

Due to the occurrence of some ovulations in case of simultaneous administration of atropine and progesterone, the estimation of maximum

time of stimulation had to be approached statistically. The incidence of failure of blockade of each time interval group was compared to the zero-hour group. The 1 and 1½-hour groups did not differ significantly, but the 2 and 2½-hour groups differed at the 5 and 1% level of probability respectively.

The same procedure used to estimate the maximum duration of the Dibenamine-blocked stimulus gave a duration of 5 hours at the 2% level of probability and 6 hours at the 1% level.

From this the conclusion was drawn that at the 1% level of probability the minimum duration of hypophyseal stimulation by the cholinergic component was 26 minutes and the upper limit close to 2½ hours.

Rothchild and Fraps (1949) found that in case of progesterone injection the hypophysis of the fowl had to remain *in situ* for at least 2 hours in order to obtain any ovulation and 4 hours to find maximal ovulatory response.

The longer duration of the adrenergic component for stimulation of the hypophysis when compared to the cholinergic component agrees with the results found in the rabbit (Sawyer, Markee, and Hollinshead, 1947; Sawyer, Markee, and Townsend, 1949; Sawyer, Markee, and Everett, 1950). In the hen both components apparently have to be effective for considerably longer time than in the rabbit. The evidence of the atropine and Dibenamine data, viewed in the light of Rothchild and Fraps (1949) experiments, seems to lead to the conclusion that the Dibenamine-blocked stimulus of the hypophysis and release of LH take place concurrently. The atropine-blocked stimulus, on the other hand, apparently has to remain effective only during the first part of the time-interval of LH release.

It may be pointed out that Thompson and Coon (1948) reported that Dibenamine did not reverse or depress the response to small or large doses of epinephrine in the fowl, with respect to the pressor response. However, Dibenamine apparently blocks the response for LH release in the progesterone-treated hen.

The experiments in which a blocking agent was given together with progesterone and in which laparotomies were performed show that the failure to palpate an egg in the oviduct was not due to internal ovulation but indeed meant failure to ovulate. Neither van Tienhoven *et al.* (1954) nor Zarrow and Bastian (1953) had excluded the possibility of internal ovulations in their experiments. Nor did either of these groups of workers prove that the effect of the blocking agent was not mediated through the ovary, although the similarities with the mechanism observed in the rat and rabbit strongly suggested that in the hen the stimulation of the hypophysis was blocked or interrupted. The experiments in which gonadotrophins were given simultaneously with the blocking agents proved this point experimentally. The results agree with those obtained in the cow,

rat, and rabbit (Hansel and Trimberger, 1951; Sawyer, Everett, and Markee, 1949; Sawyer, Markee, and Townsend, 1949).

SUMMARY

In progesterone-induced ovulation of the hen there seems to be a difference in duration of the adrenergic and the cholinergic component involved in hypophyseal stimulation.

The minimum time of stimulation of the cholinergic component is about 26 minutes, the maximum time approximately 2½ hours.

The reported data indicate a concurrent stimulation and release of LH from the hen's anterior pituitary gland.

Neither Dibenamine nor atropine affects the hen's oviduct prior to ovulation in such a manner that internal ovulations occur.

Neither Dibenamine nor atropine interfered with exogenous gonadotrophin-induced ovulations.

Acknowledgments

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SODIUM FLUOROACETATE DIABETES: CORRELATIONS BETWEEN GLYCEMIA, KETONEMIA AND TISSUE CITRATE LEVELS

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IN A previous report (Engel, Hewson and Cole, 1954) it was shown that the administration of sodium fluoroacetate (SFA) to the fasted rat resulted in the development of a striking hyperglycemia and ketonemia beginning in a few hours and most apparent in 24 hours. However, no observations were made beyond the latter time. The ketonemia and hyperglycemia were explained in terms of primary and secondary responses to the block in the tricarboxylic acid cycle caused by the SFA. Ketonemia, thus, was considered a consequence of failure of oxidation of acetate as well as of acetoacetate itself with resultant accumulation of the latter compound. The hyperglycemia was interpreted as secondary to a decrease in hexokinase activity resulting from a declining supply of high energy phosphate from the impaired Krebs cycle. In addition the possibility was considered that Krebs cycle blockade might interfere specifically with insulin synthesis by the beta cells of the islets of Langerhans and hence insulin deficiency might be a factor in the diabetes.

The purpose of the present study was to characterize SFA diabetes more completely in terms of its duration and to correlate the latter with the metabolic hallmark of sodium fluoroacetate intoxication, tissue citrate accumulation (Chenoweth, 1949; Peters, 1952). It was found in general that the evidences of diabetes and increased tissue citrate concentrations paralleled each other, all values having returned to essentially normal levels in animals surviving 64 hours. Attempts to induce permanent diabetes were unsuccessful, although it is noteworthy that a single animal survived for two weeks with hyperglycemia and ketosis. In addition, observations on the effects of cortisone acetate on tissue citrate levels in SFA poisoned rats are described.

METHOD AND MATERIALS

Male rats of the Vanderbilt strain, weighing between 250 and 400 gm. were used. The diet and general handling of the animals were as in the previous report (Engel, Hewson

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and Cole, 1955) except that no anesthesia was used. Sodium fluoroacetate (Monsanto) was injected intraperitoneally as a freshly prepared 0.06% saline solution at a dose of 0.6 mg./100 gm. body weight. This dose is somewhat smaller than that employed in the previous study and was chosen to allow for a better survival.

Tail blood was analyzed for ketone bodies by a modification of the method of Greenberg and Lester (Greenberg and Lester, 1944; Lester and Greenberg, 1948; Werk, Hamrick, McPherson, Myers and Engel, 1955) and for sugar according to Somogyi (1945). Tissue citrate concentrations were determined by the method of Ettinger, Goldbaum and Smith (1952).

The rats were sacrificed by decapitation after bleeding and the tissues immediately dissected and quickly frozen by dropping them into liquid air. The tissues were stored in the frozen state in the deep freeze until citrate analyses were performed. For the latter they were homogenized in water in the cold in a Potter-Elvehjem homogenizer. Protein was precipitated with tungstic acid. In the case of muscle and heart, which were not readily homogenized, the stored frozen tissue was hardened by pressing between two blocks of dry ice and was converted into a powder by pounding with an aluminum cylinder rod on an aluminum anvil. The powder was then added to a tared tube of cold water and weighed. Preliminary observations indicated that there was no detectable loss of citrate when tissues were stored for weeks in the deep freeze.

RESULTS AND DISCUSSION

Duration of hyperglycemia and hyperketonemia: Figure 1 records the effects of a single intraperitoneal injection of 0.6 mg. SFA/100 gm. in fasted rats. SFA was injected at the end of a 24 hour fast and blood sugar

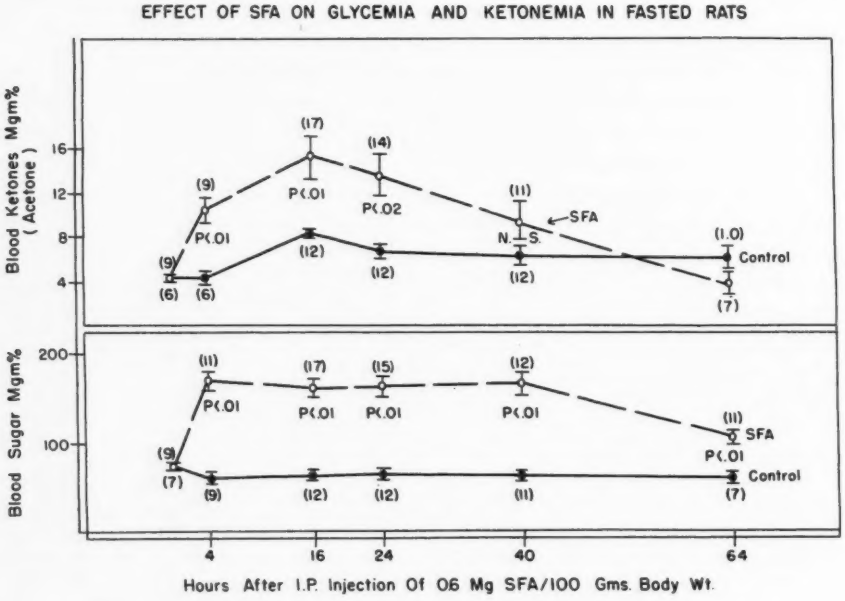

FIG. 1. Glycemia and ketonemia following intraperitoneal injection of 0.6 mg. SFA/100 grams in fasted rats.

and ketone body levels estimated at 4, 16, 24, 40 and 64 hours after injection. Significant hyperglycemia was apparent within four hours and persisted unchanged for 40 hours. By 64 hours the blood sugar elevation had fallen off substantially, but the level was still significantly higher than in the controls ($p < .01$). Persistent hyperglycemia during fasting is, of course, characteristic of diabetes mellitus. Ketonemia was also well established by four hours and reached its peak in 16 hours. Blood ketones were still significantly elevated at 24 hours but not at 40 or 64 hours. Many animals died after blood sampling at 40 and 64 hours and their clinical appearance suggested peripheral circulatory failure. This complicates the interpretation of the ketone values since it has been shown that peripheral circulatory failure inhibits fasting ketosis in the rat (Engel and Hewson, 1953).

A single animal that received 0.8 mg SFA/100 gm. survived for 14 days in the fasting state. During the entire period it exhibited hyperglycemia and ketonemia, blood sugar and ketone levels on the 13th and 14th days being 294 and 245 mg. % and 41.9 and 30.6 mg. % respectively. Although all subsequent attempts to reproduce this experience have been unsuccessful it is our opinion that this single observation is of considerable significance. It suggests that under the proper experimental conditions permanent diabetes mellitus might be produced with SFA and as such the possibility of pancreatic islet cell damage must be entertained as a basis for even the temporary diabetes.

Another experiment was performed in fed animals (Fig. 2). Since the food intake of the SFA poisoned rats was known to be low a control series was run in which the food intake was restricted to that consumed by the poisoned rats. The results in Figure 2 indicate that the food restriction per se had no notable influence on either glycemia or ketonemia. Again the SFA treated rats exhibited hyperglycemia but unexpectedly, it was not as sustained as in the previous experiment with fasted rats. Despite eating, on the other hand, ketonemia was as striking as in the fasted rats and was still significantly apparent at 40 hours.

Correlations between tissue citrate and blood ketone and sugar levels: The occurrence of hyperglycemia and ketosis during both the fasting and the fed states clearly identifies the metabolic disturbance of SFA poisoning in the rat as diabetes mellitus. It remained to be established whether or not there was a close correlation between the blood changes indicative of diabetes mellitus and the stigma of fluoroacetate poisoning, elevated tissue citrate concentration. This is of some importance in view of the possibility of permanent pancreatic damage suggested by the single observation, noted above, of a rat which survived 14 days with hyperglycemia and ketosis.

Figures 3, 4 and 5 illustrate the results of tissue citrate and blood ketones and sugar levels at 4, 16, 24, 40 and 64 hours of poisoning with 0.6

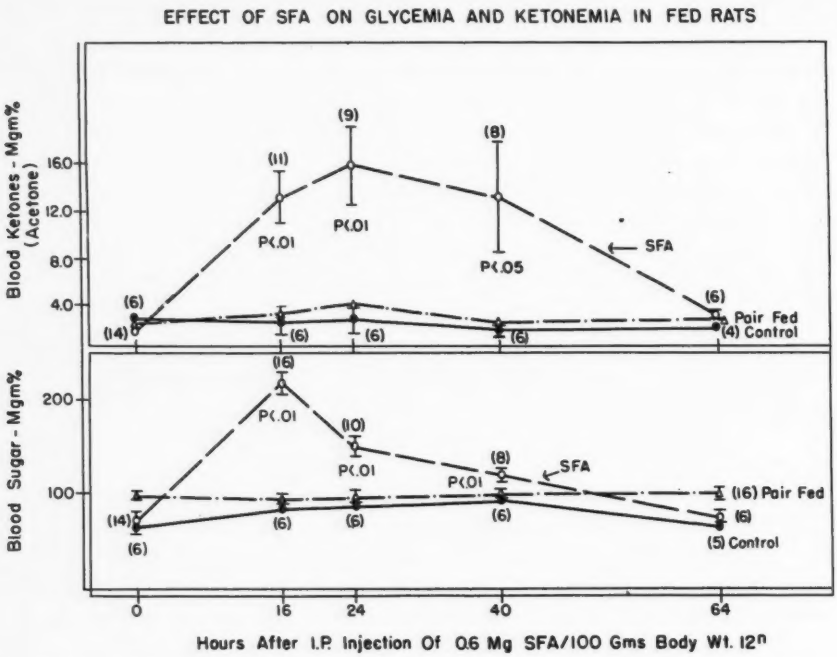
EFFECT OF SFA ON GLYCEMIA AND KETONEMIA IN FED RATS
Blood Ketones - Mgm% (Acetone)
Blood Sugar - Mgm%
SFA
Pair Fed
Control
P<.01
P<.05
Hours After I.P. Injection Of 0.6 Mg SFA/100 Gms Body Wt. 12ⁿ

FIG. 2. Glycemia and ketonemia following intraperitoneal injection of 0.6 mg. SFA/100 grams in fed rats compared to ad libitum and pair-fed controls.

mg. SFA/100 grams compared to corresponding untreated controls fasted at the same time. Examination of Figure 5 shows that less ketosis but more hyperglycemia developed in this experiment compared to the similar study depicted in Figure 1. The precise reason for this difference is not apparent unless it be due to the fact that in the former experiment rats weighing between 200 and 300 gm. were used while in the latter 300–400 gm. rats were utilized.

The magnitude of the tissue citrate accumulation and its duration in different tissues corresponded reasonably well with other reports in the literature (Lidenbaum *et al.*, 1951, Potter *et al.*, 1951). Furthermore, there was an over-all good gross correlation between the duration of hyperglycemia and tissue citrate accumulation in most tissues. Greatest citrate levels were achieved in heart, pancreas, kidney and spleen and least in liver and muscle. In the heart (Fig. 3) the peak citrate level occurred at 16 hours, but a significant elevation still was demonstrable at 40 hours. Liver showed a peak at 24 hours but thereafter there was no difference from the control (Fig. 3). Kidney and spleen citrate levels (Fig. 4) were maximal at four hours but remained significantly elevated longer, i.e. 64 hours in the case of kidney and 40 hours in the case of spleen. Muscle showed only

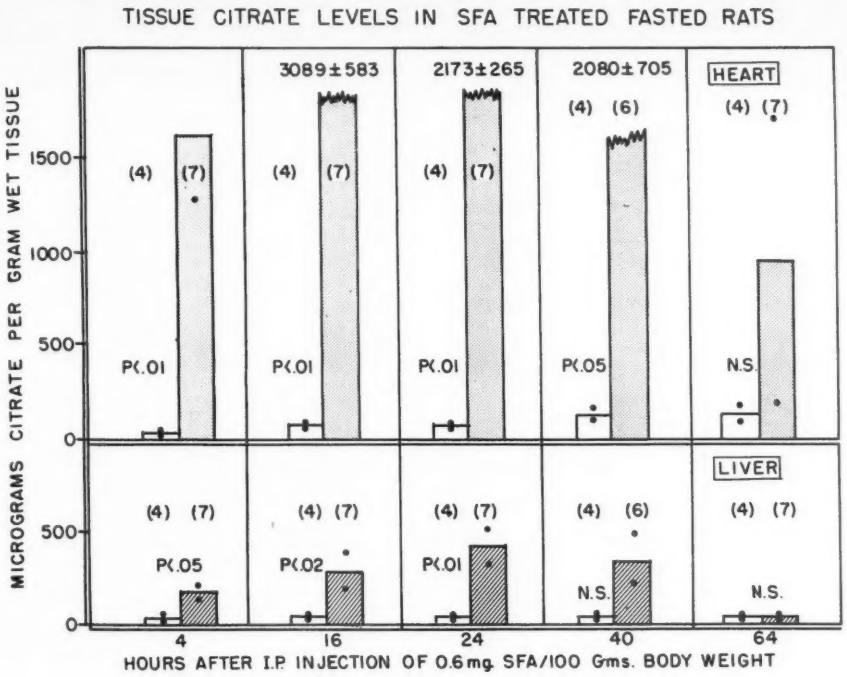

FIG. 3. Heart and liver citrate levels in SFA treated rats (shaded column) compared to untreated controls.

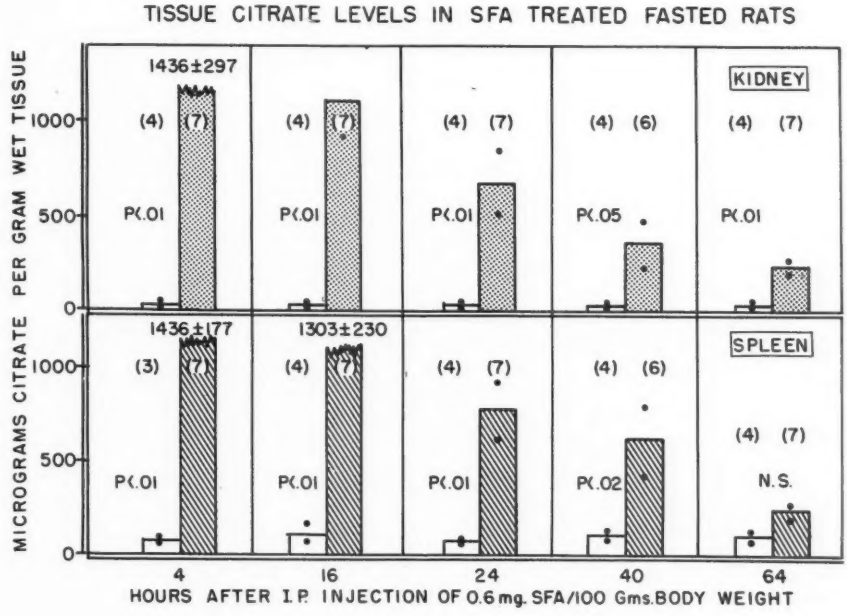

FIG. 4. Kidney and spleen citrate levels in SFA treated rats compared to untreated control.

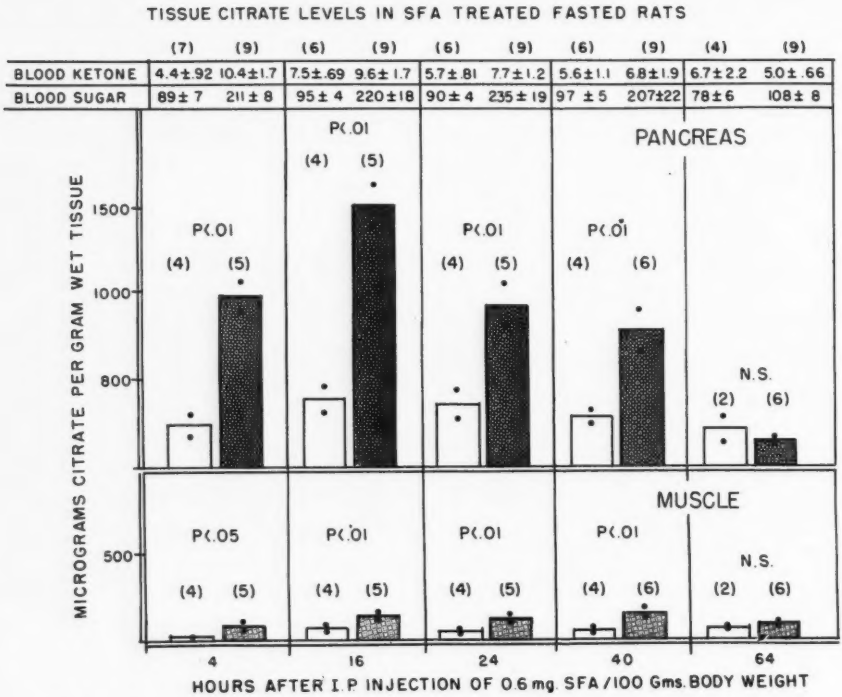

FIG. 5. Pancreas and muscle citrate and blood ketone and sugar levels in SFA treated rats compared to untreated controls.

small increments in citrate but these were significant through 40 hours (Fig. 5). Pancreas citrate levels peaked at 16 hours and were still highly significantly elevated at 40 hours. Attempts to interpret the latter change in terms of specific pancreatic damage with insulin deficiency must be tempered by the knowledge that islet tissue represents less than 1% of the weight of the gland. Taken as a whole, the relatively good correlation between the degree and duration of the diabetes and the tissue citrate elevations indicates that the basic chemical defect of fluoroacetate poisoning, blockade of the Krebs citric acid cycle, in all likelihood plays a primary role in the diabetes. The results, however, do not rule out the possibility of the same defect producing a specific failure in insulin production. This possibility is under further investigation in this laboratory.

Influence of cortisone acetate on the diabetes and tissue citrate levels. It was previously reported that pretreatment for 48 hours with 5 mg. of cortisone acetate daily intramuscularly prevented the ketosis but not the hyperglycemia of SFA poisoning (Engel, Hewson and Cole, 1955). The mechanism by which cortisone induced its effect was by no means clear but it was suggested that it might have done so by increasing the avail-

TABLE 1. EFFECT OF CORTISONE ON TISSUE CITRATE AND BLOOD KETONES AND SUGAR LEVELS IN SFA POISONED RATS

	Tissue citrate in micrograms/gm. wet tissue												Mg.% in blood			
	Liver		Spleen		Kidney		Pancreas		Heart		Muscle		Ketones		Sugar	
Hours after SFA	4	24	4	24	4	24	4	24	4	24	4	24	4	24	4	24
Control	30 ± 9 (4)	21 ± 2.5 (7)	75 ± 12 (3)	87 ± 19 (4)	26 ± 11 (4)	23 ± 10 (4)	246 ± 70 (4)	351 ± 91 (4)	34 ± 5 (4)	62 ± 13 (4)	13 ± 1.2 (4)	36 ± 5 (4)	4.40 ± 0.92 (7)	5.70 ± 0.81 (6)	89 ± 7 (7)	90 ± 4 (6)
SFA	168 ± 54 (7)	428 ± 91 (7)	1436 ± 297 (7)	734 ± 147 (7)	1436 ± 177 (7)	690 ± 168 (7)	982 ± 90 (5)	937 ± 102 (5)	1624 ± 427 (7)	2173 ± 265 (7)	76 ± 23 (5)	103 ± 5 (5)	10.41 ± 1.7 (9)	7.70 ± 1.2 (9)	211 ± 19 (9)	235 ± 19 (9)
Cortisone	26 ± 8 (5)	13 ± 3 (5)	48 ± 8 (5)	62 ± 11 (5)	24 ± 8 (5)	12 ± 3 (5)	122 ± 14 (5)	191 ± 26 (5)	27 ± 5 (3)	26 ± 5 (4)	18 ± 4 (5)	23 ± 4 (4)	2.44† ± 0.34 (5)	3.69† ± 0.36 (5)	91 ± 2 (5)	95 ± 6 (5)
Cortisone and SFA	494 ± 123 (7)	293 ± 80 (9)	1098 ± 123 (7)	675 ± 54 (10)	87† ± 169 (8)	712 ± 165 (16)	733† ± 63 (8)	682 ± 71 (10)	1208 ± 158 (8)	1808 ± 210 (10)	112 ± 7 (8)	102 ± 17 (10)	4.10* ± 0.50 (8)	4.42° ± 0.50 (10)	231 ± 40 (8)	299† ± 23 (10)

* $p < .01$
° $p < .02$
† $p < .05$.

ability of Krebs cycle intermediaries from dicarboxylic and other amino acids arising from protein catabolism thereby allowing for greater oxidation of acetate. In light of this observation it was deemed of interest to examine the effect of this hormone on tissue citrate levels in SFA treated rats. The experiment was performed exactly as before except that groups of animals were sacrificed at 4 and 24 hours after SFA poisoning and no nembutal anesthesia was used. The data are recorded in Table 1. The data on control and SFA poisoned rats not receiving cortisone are the same as recorded in Figures 3, 4 and 5. Compared to untreated animals ketonemia was suppressed while hyperglycemia persisted in the cortisone treated rats poisoned with SFA. However the degree of inhibition of ketosis at 24 hours after SFA in this experiment is not as apparent as in the previous study since, as noted above, the SFA poisoned rats of this group developed less ketosis at 24 hours than usual. Hyperglycemia was greater ($p < .05$) in the cortisone SFA group than in the rats receiving only SFA.

Cortisone treatment had no effect on the citrate concentration of the tissues of unpoisoned animals. Results in the SFA poisoned rats were somewhat more irregular, but in general significant differences in tissue citrate concentrations did not occur except in kidney and pancreas where the levels were slightly lower ($p < .05$) in the cortisone treated rats at 4 but not at 24 hours. The failure of cortisone to modify tissue citrate levels while at the same time abolishing ketosis is consistent with the mechanism of action previously proposed. It is comparable to the situation whereby glycerol monoacetate abolished the convulsions and corrected the EEG changes in SFA poisoned dogs without modifying the brain citrate level (Chenoweth *et al.*, 1951; Kandel and Chenoweth, 1952). Glycerol monoacetate also prevents the ketosis in SFA poisoned rats (Engel, Hewson and Cole, 1954). In both cases it is presumed that an increased supply of Krebs cycle constituents is made available to by-pass the blockade.

SUMMARY

1. Sodium fluoroacetate was administered intraperitoneally to fasted and fed rats. Hyperglycemia and ketosis developed within four hours. Ketosis persisted between from 24–40 hours and hyperglycemia from 40–64 hours in both groups.

2. A single SFA poisoned rat exhibited a prolonged survival, dying on the 14th day of fasting with marked hyperglycemia and ketosis.

3. The citrate content of heart, liver, spleen, kidney, pancreas and gastrocnemius muscle was estimated in SFA poisoned rats and found to correlate reasonably well with the hyperglycemia and ketosis. With the exception of spleen, citrate levels were all back to normal by 64 hours.

4. Pretreatment with cortisone acetate prevented the ketosis of SFA poisoning and increased the hyperglycemia but did not modify the tissue citrate concentrations.

5. These data are interpreted to indicate that the Krebs cycle blockade in the tissues in general plays a primary role in the etiology of SFA diabetes. The single observation of persistent diabetes, however, leaves open the possibility of specific damage to insulin producing cells as a factor in the diabetes.

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INHIBITION OF AGGRESSIVENESS BY OVARIAN HORMONES[1]

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INTRODUCTION

A CONSIDERABLE body of experimental and observational evidence indicates that the social behavior of animals is influenced by gonadal hormones (Beach, 1948). In many species androgen is associated with aggressive activity (Collias, 1944). Testicular hormone has been found to increase the incidence of fighting behavior in male reptiles (Evans, 1946; Noble and Greenberg, 1941), birds (Davis and Domm, 1941; Emlen and Lorenz, 1942) and mammals (Beeman, 1947). Androgen also stimulates aggressiveness in the females of some mammalian species (Huffman, 1941). Some animals establish and maintain stable social orders which are characterized by differential dominance status for each invididual, and the administration of androgenic hormones tends to increase the dominance ranking of treated individuals although species differences in this respect are marked (Birch and Clark, 1950; Mirsky, in press).

In contrast to androgen, estrogen has been regarded as an inhibitor of aggressive behavior although this is not true in all cases (Birch and Clark, 1950; Mirsky, in press). Administration of estrogen to male birds results in a decrease in fighting (Davis and Domm, 1943). The intact female American chameleon tends to fight much less than the male but she becomes quite aggressive after ovariectomy (Evans, 1936).

Females of several mammalian species including the opossum and the shorttailed shrew will tolerate the attentions of the male during the period of estrus, but at all other times the female will severely wound any male who approaches her (Hartman, 1945; Pearson, 1944). A comparable situation appears to obtain in the case of the golden hamster (*Cricetus auratus*). Adult female hamsters are larger than males and usually prove victorious in any aggressive encounter. Preliminary observations by W. L. Stoops and E. L. Rolett in the Yale Psychological Laboratory revealed that the aggressiveness of the female varies during different phases of the reproductive cycle. Stoops and Rolett found that there is a marked decrease in fighting behavior when the female is in estrus. The present experiment

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represents an extension and elaboration of this earlier work and had as its objective the correlation of varying degrees of aggressiveness with differences in the presence or absence of ovarian hormones.

PROCEDURE

Selection and Maintenance of Subjects

Eighteen female hamsters from 4 to 6 months of age were observed with males for the occurrence of fighting behavior. The 9 females showing the most frequent and vigorous fighting were chosen as subjects. Throughout the experiment these animals were kept in individual cages and provided with laboratory chow and water *ad libitum*. The light-dark cycle in the experimental room was reversed so that the hamsters were exposed to artificial illumination during the night, and the room was darkened during the day. All tests were conducted during the dark phase of the reversed cycle.

Males used as stimulus animals were of the same strain and age as the experimental females.

Testing Methods

During observation periods the male-female pairs were put together in a cage of the same dimensions and construction as the individual living cages. The experimental room was darkened and the observation cage was illuminated by a 25 watt bulb suspended directly above it. Before each test the female was placed in the observation cage and left alone for 30 seconds, at the conclusion of which time the stimulus male was introduced. Each female was tested with the same partner throughout the experiment, and males were vasectomized before experimentation to prevent the occurrence of pregnancy.

Pairs were left together for a total of 10 minutes during which time the experimenter recorded the latency,[2] duration and frequency of several discrete types of behavior. These items were as follows: (1) fights, which consisted of one or both animals grasping the opponent with all four legs, squealing, rolling about the cage floor, and biting which often resulted in severe wounds; (2) pins by the female, in which she forced and held the male against the wall or floor of the cage and usually bit him though not severely; (3) spars, in which both animals reared up on their hind legs and struck out with the forepaws or pushed or leaned against one another;[3] (4) copulations, in which the male mounted the female; and (5) assumption of lordosis or the mating position by the female.

After an observer had become experienced in recognizing and differentiating these reactions it proved possible to keep a running record of every test. In preliminary phases of the experiment two observers scored the same tests independently and then compared their records. Although no statistical treatment of the results was attempted, it was apparent that our method of recording interaction in the test situation was highly reliable.[4]

Schedule of Tests, Operations and Hormone Treatment

All females were observed in 10 successive, daily tests with males, and they were then randomly divided into an experimental and a control group. The 6 experimental females

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[2] Time from the beginning of the test to the first occurrence of the behavior in question.

[3] Spars were invariably so brief that it was not practical to measure their duration.

[4] The authors gratefully acknowledged the assistance of Mr. Julian Jaynes in devising and perfecting this method of scoring behavior.

were ovariectomized, and the 3 control cases were subjected to a sham operation involving anesthesia and all of the stages of surgery save removal of the ovaries. Testing was resumed 6 days after the operation.

The control females were observed in 17 daily, postoperative tests in the course of which they continued to show normal estrus cycles. Experimental females were given 10 daily tests after operation and in no instance was any estrus behavior observed. At the conclusion of the tenth postoperative test each experimental hamster was given an injection of .225 mg. of estradial benzoate.[5] Twenty-three hours later another fighting test was conducted and it was followed promptly by a second injection of the same amount of estrogen. Daily tests and hormone injections continued until a total of 6 injections had been administered. Four hours before the next test each female was injected with .025 mg. of progesterone. During the test following progesterone treatment all animals were sexually receptive as shown by their ready display of lordosis. One day later they were tested again and were found to have passed out of artificially induced estrus.

Two weeks after the test that immediately followed progesterone treatment, 2 of the spayed females were observed in a series of 3 successive, daily fighting tests. Four hours before the second and third of these tests animals were injected with .025 mg. of progesterone.

RESULTS

Reliability and Validity of Behavioral Measures

In the course of the experiment it became obvious that fighting and pinning of the male by the female were related activities, tended to vary together and were clearly influenced by the physiological condition of the female. Sparring appeared to belong in a separate category for it was only slightly affected by changes in the female's condition and was the only measure to undergo progressive change in successive stages of the investigation. Comparisons between tests run early and late in the experiment showed that the latency of sparring increased, while the average frequency of sparring encounters decreased. In other words, repeated testing brought about a decrease in the tendency to spar, although this effect was not observed in connection with any other behavioral measure. Because it was only slightly affected by the female's hormonal condition, and because it tended to decrease with increasing experience in the testing situation whereas other measures did not, sparring was judged to be an invalid indicator of aggression and we have not included the records of sparring in the following analysis of results.

Assuming that fighting and pinning might be valid measures of aggressiveness, we were still concerned with their statistical reliability, since an unrealiable or inconsistent measure is of little use in the analysis of behavior. To test the consistency of the behavior scores we calculated the rank-order correlations between scores recorded during the odd and during

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[5] The estrogen and progesterone used in this work were generously supplied by Dr. Edward Henderson, Schering Corporation.

TABLE 1. RELIABILITY OF DIFFERENT BEHAVIORAL MEASURES BASED ON RANK-ORDER CORRELATIONS

Average scores	Behavioral measure	
	Fights	Pins
Frequency per test	+.88	+.88
Latency	+.08	+.60
Duration	+.73	+.02

the even-numbered tests occurring when the females were nonreceptive and intact. This included a total of 59 tests for the 9 pairs of animals. The resulting coefficients are shown in Table 1.

It will be noted that the average frequencies of fights and pins were reasonably consistent from test to test, indicating that individual differences were stable and reliable. With the exception of the duration of fights, other correlations were not high enough to be impressive, although they might have been more reliable if a larger N had been tested. In general, these correlations suggest that at least as far as frequency is concerned our measures reflected consistent tendencies toward aggressive behavior, and that they could be used to establish a normal baseline with which the effects of experimental variables might be compared.

General Effects of Feminine Receptivity on Aggression in Nine Pairs

Data summarized in Table 2 are based upon the behavior of 9 male-female pairs in 140 tests. The females were sexually receptive during 48 of these tests and nonreceptive in the remaining 92. We have included the complete records of the 6 experimental and 3 control pairs. Control data include tests made before and after the dummy operation because this was without effect upon behavior. Females that were subjected to the sham ovariectomy continued to show regular estrus cycles and their aggressive behavior waxed and waned in a completely normal manner.

The table shows the percentage of tests in which each type of behavior occurred, and the number of pairs showing the response. Under the conditions of this experiment, fighting almost inevitably occurred if the female was nonreceptive, and it was completely inhibited as long as she was in

TABLE 2. PRESENCE OR ABSENCE OF AGGRESSIVE BEHAVIOR IN 140 TESTS OF NINE MALE-FEMALE PAIRS

Behavioral measure	Condition of female			
	Non-receptive (92 tests)		Receptive (48 tests)	
	No. of females responding	Per cent of tests	No. of females responding	Per cent of tests
Fighting	9	98	0	0
Pins	9	73	3	8

TABLE 3. FREQUENCY MEASURES OF AGGRESSIVE BEHAVIOR ON THE SIX EXPERIMENTAL PAIRS

Behavioral measure	Condition of female				
	Intact		Spayed		
	Non-receptive (36 tests)	Receptive (23 tests)	Untreated (60 tests)	Given estrogen (30 tests)	Given estrogen plus progesterone (6 tests)
No. Females Showing					
Fighting	6	0	6	6	0
Pins	6	2	6	6	1
Per Cent of Tests with:					
Fighting	97	0	95	100	0
Pins	67	12	73	87	17
Average Frequency per Positive Tests*					
Fighting	3.8	0	2.6	4.9	0
Pins	4.7	1.0	1.8	3.4	1.0

* "Positive" tests are those in which the response in question occurred at least once.

estrus. Pinning responses occasionally were shown by receptive females, though much less often than by females not in estrus.

Frequency of Aggressive Behavior in Experimental Pairs

In Table 3 are shown frequency scores of the 2 types of aggressive behavior as displayed by the 6 experimental pairs under different conditions. Table 4 presents time scores for the same tests. The data have been categorized in terms of the physiological condition of the female at the time of testing.

Females intact and receptive vs intact and nonreceptive. Fighting was shown by all pairs when the female was nonreceptive and by none when she was in heat. It occurred in 97% of the 36 "nonreceptive tests." There

TABLE 4. TIME MEASURES ON SIX PAIRS

Mean score in seconds	Condition of female				
	Intact		Spayed		
	Non-receptive (36 tests)	Receptive (23 tests)	Untreated (60 tests)	Given estrogen (30 tests)	Given estrogen plus progesterone (6 tests)
Latency of:					
Fight	204	—	258	236	—
Pins	200	281	249	168	1 (1 test)
Duration of:					
Fight	2.3	—	2.5	2.8	—
Pins	8.2	1.5	5.4	7.3	7.0 (1 test)

were on the average 3.8 fights per test during those tests in which this behavior appeared. The average latency of fights was 204 seconds, and their average duration was 2.3 seconds.

All females pinned the male in some of the nonreceptive tests, but this response was shown by only 2 females when they were receptive. It occurred in 67% of the nonreceptive tests and 12% of the tests during which the females were in heat. Although some pinning was exhibited by estrus females, the strength of the behavior was reduced, as can be deduced from the fact that in receptive tests its frequency was lowered, its latency was longer and its duration was shorter than in tests when the female was not sexually receptive.

Females intact and nonreceptive vs spayed and untreated. When the females were ovariectomized and not given hormone treatment, the behavior was comparable to that occurring in pre-operative, nonreceptive tests. Table 3 shows that the percentage of tests in which each of the two types of aggressive behavior occurred was quite similar under the two conditions. There are, however, several indications that the ovariectomized hamster may be slightly less aggressive than the intact female in diestrus. As seen in Table 3, although fighting and pinning occurred in both conditions, the average frequency of both responses was lower when the female had been spayed. In the case of frequency of fights, the difference is significant at the .03 level of confidence.

Table 4 shows that differences in time scores support the suggestion that aggressiveness is slightly reduced by removal of the ovaries. In all behavioral measures latencies were increased by ovariectomy.

Females spayed and untreated vs spayed and given estrogen. Table 3 reveals that administration of estrogen to spayed females was followed by an increase in the percentage of tests in which aggressive behavior occurred. Furthermore, during those tests in which fighting and pinning appeared, their average post-operative frequency was increased by estrogen. Probability values for differences in mean frequencies were as follows: fighting, .04; pins by female, .03.

Data summarized in Table 4 also indicate some intensification of aggressive tendencies in ovariectomized females consequent to estrogen treatment. The latencies of both aggressive measures were shorter after estrogen injections, although the change was statistically significant only in the case of pins by the female ($p = .01$). The same table shows that the average duration of aggressive encounters tended to increase as a result of estrogen treatment. In the case of pins by the female the increase was statistically significant at the .05 level.

Females spayed and untreatee vs spayed and given progesterone. The effects of administering progesterone to spayed females were tested on only 2 pairs and the average scores are therefore not included in the tables. Careful inspection of the records revealed no effect of the hormone. Aggressive

behavior was quantitatively and qualitatively the same after progesterone injection as it had been in post-operative tests without hormone treatment.

Females spayed and brought into heat. Although neither estrogen nor progesterone alone will produce sexual receptivity in the ovariectomized hamster, a series of estrogen injections followed by one injection of progesterone induces all the behavioral signs of estrus, and this condition persists for several hours.

Tables 3 and 4 show that when our experimental females were brought into heat in this manner they were no more and perhaps even less aggressive then they had been during normal estrus. The results are not presented separately in the tables, but tests conducted 24 hours after the induced estrus revealed that the effects of the exogenous hormones had been dissipated and aggressiveness had returned to pre-treatment levels.

Relation of Aggressiveness to Sexual Behavior

In an attempt to determine possible relationships between aggressiveness and tendencies toward sexual behavior, we calculated the rank-order correlations between various measures of aggression with the performance of each pair during those tests when the female was unoperated and receptive. The average frequencies of fights and pins were correlated with mean frequency of mounting responses during receptive tests. The resulting coefficients were all negative but of low value, ranging from $-.09$ to $-.24$. The correlation between pins and frequency of "copulations," (i.e. mounts which resulted in intromission) was also low and negative. The frequency of fights and of copulations correlated positively at the level of $+.34$, which is not statistically significant. These findings give us no reason to believe that there is any reliable relationship between the amount of aggressive behavior which a pair shows when the female is nonreceptive, and the amount of sexual activity that occurs when she is in heat.

DISCUSSION

The foregoing results establish the fact that in the female hamster aggression and sexual receptivity are negatively related. In this species, fighting and copulating are mutually exclusive categories of response. Milder forms of aggression, such as pinning another animal, may occur when the female is in estrus, but they are much less frequent and intense at that time.

It is further apparent that the mechanisms underlying this negative relationship are endocrinological. The same combination of ovarian hormones that elicits receptivity tends to inhibit aggressive behavior.

Finally, there is some suggestion that estrogen produces an increase in aggressiveness. This is indicated by two lines of evidence. First, spayed females became slightly more aggressive when given several injections of estrogen. Second, untreated spayed females were somewhat less aggressive

than intact animals in the nonestrus condition. Many of the "nonreceptive" tests conducted before operation probably occurred shortly before estrus, when maturing follicles had already begun to secrete estrogen but before there were corpora lutea to produce progesterone. It is our belief that the difference in aggressiveness of nonreceptive, intact animals and spayed females was due to the presence of estrogen in the unoperated females.

SUMMARY

Nine oppositely-sexed pairs of golden hamsters were observed in a series of standardized tests for aggressive behavior and mating reactions. It was found that fighting never occurred when the female was sexually receptive and was almost invariably present when she was not in heat. Less intense forms of aggressive interaction were observed in "receptive" tests, but they were less frequent and less prolonged than in tests during which the female was nonreceptive.

Six females were ovariectomized and 3 were subjected to sham surgery involving all steps in the operation except actual removal of the ovaries. The dummy operation had no effect upon behavior. Females undergoing this treatment continued to display regular estrus cycles and to be docile when receptive and aggressive at all other times.

Ovariectomized hamsters were continuously aggressive although somewhat less so than intact animals in diestrus. Six daily injections of estrogen were followed by a slight increase in aggressiveness. A single injection of progesterone following the estrogen treatment brought the spayed females into heat, eliminated fighting, and drastically reduced other forms of aggression. The effects of the exogenous hormones were short-lived, and 24 hours later all females were nonreceptive and very aggressive. Progesterone injections that were not preceded by the administration of estrogen had no influence upon the behavior of spayed females.

These results make it clear that there is a negative relationship between sexual receptivity and aggressiveness in the normal female hamster, and that this relationship depends in large measure upon endocrine secretions. The ovarian hormones that render the female receptive also serve to inhibit her tendency to attack the male.

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A STUDY OF THE SEX DIFFERENCE IN THE NUMBER OF CIRCULATING EOSINOPHILS IN THE GUINEA PIG

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REPORTS on alterations in the number of circulating eosinophils after various experimental procedures are frequently based on samples of varying weight range and different sex. There is some question as to the validity of such pooled populations in view of studies on the guinea pig indicating a sex difference in eosinophil count (Dworetsky, Code and Higgins, 1950; Dew and Code, 1951), and on the mouse (Speirs, 1952) indicating an increase in number of eosinophils with age. In the light of these findings, data on our colony have been recalculated for separate sexes and for various weight ranges.

EXPERIMENTAL PROCEDURE

The 350–600 gram guinea pigs used in these experiments are from our colony, started in 1950 with 4 females and 2 males purchased from a commercial breeder. All counts were made between 9 and 10:30 A.M. to eliminate the possible effect of diurnal fluctuations (Best and Samter, 1951). Where more than one count is reported for the same animal the determinations were made at least one week apart. Precautions necessary for consistent blood counts on this highly excitable animal, and the method of counting are discussed elsewhere (Schweizer, 1953).

OBSERVATIONS

Relation of Body Weight to Age

Some of the comparisons in this report will be expressed in terms of body weight as a criterion for age. Since this is valid only if the rates of growth for the sexes are similar, the body weights of males and females at various ages are compared. The data summarized in Table 1 indicate that there is no appreciable difference in the body weight of males and females of the same age for the intervals studied (2 days to 5 months).

White Blood Cells

The number of white blood cells in 350–600 gram male and female guinea pigs is compared in Table 2. The mean number of eosinophils in the normal female (26 counts on 17 animals) is 89% higher than in the normal male (31 counts on 14 animals). The inclusion of more than one count on

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TABLE 1. RELATION OF BODY WEIGHT TO AGE

Age	Males		Females	
	No. of animals	Body weight, gms. (range)	No. of animals	Body weight, gms. (range)
2 days	12	79.2 (60–130)	7	88.3 (70–95)
1 week	5	145.0 (125–240)	7	111.4 (80–130)
2 weeks	7	165.0 (155–225)	7	157.9 (130–185)
1 month	18	270.0 (210–395)	22	248.4 (185–300)
2 months	13	398.1 (320–470)	13	367.7 (300–460)
3 months	12	480.9 (420–555)	11	492.8 (425–555)
4 months	14	562.5 (525–660)	5	536.0 (500–625)
5 months	8	593.4 (500–640)	6	563.3 (525–610)

some of the animals is justified by the fact that the mean for the initial counts on 14 males and 17 females is not significantly different from the mean of 31 and 26 counts, respectively, on the same group.

This sex difference in number of eosinophils disappears after removal of the pituitary. Both sexes have a higher count after operation, but the increase in the hypophysectomized male is greater (+200%, $P < .01$) than in the hypophysectomized female (+62.8%, $P = .05$).

There is no sex difference in the number of mononuclear cells in the normal guinea pig, but after hypophysectomy the count in the female is higher than in the male. This difference arises because the number of mononuclear cells falls after operation in the male (−20%, $P < .01$) but remains essentially unchanged in the female.

TABLE 2. WHITE BLOOD CELLS

	Males			Females		
	No. of animals	No. of counts	Cells/mm.³ ±S.E.	No. of animals	No. of counts	Cells/mm.³ ±S.E.
			Eosinophils			
Normal	14	31	101 ±18.7	17	26	191 ±34.7*
	14	14	93 ±19.7	17	17	211 ±40.0*
Hypophysectomized	11	29	303 ±53.8	8	15	311 ±58.1
	11	11	340 ±79.7	8	8	328 ±44.3
			Mononuclear Cells			
Normal	14	31	5489 ±244.4	17	26	5633 ±217.6
	14	14	5720 ±308.1	17	17	5797 ±270.7
Hypophysectomized	11	29	4290 ±272.2	8	15	5853 ±326.9†
	11	11	4134 ±390.9	8	8	5563 ±471.4†
			Polymorphonuclear Cells			
Normal	14	31	3972 ±294.6	17	26	3463 ±377.4
	14	14	4007 ±340.5	17	17	3458 ±329.2
Hypophysectomized	11	29	3448 ±304.6	8	15	3283 ±289.7
	11	11	3006 ±321.2	8	8	3506 ±185.8

* Significant difference from males, $P = .02$.
† Highly significant difference from males, $P < .01$.

TABLE 3. RELATION OF NUMBER OF EOSINOPHILS TO BODY WEIGHT (AGE)

Body weight, gms. (approximate age)	Males		Females	
	No. of animals	Cells/mm.^{3} ±S.E.	No. of animals	Cells/mm.^{3} ±S.E.
301–400 (2 months)	3	84	3	140
401–500 (3 months)	11	73 ± 13.0	7	139 ± 23.4*
501–600 (4 months)	15	82 ± 17.3	7	192 ± 26.5*

* Highly significant difference from males, $P < .01$.

Male and female guinea pigs have no significantly different polymorphonuclear cell counts when either in the intact or hypophysectomized state.

Relation of number of Eosinophils to Body Weight (Age)

The animals used in these experiments were approximately 2–4 months old. The data summarized in Table 3 show that within this age range the eosinophil count does not alter significantly with age, and that within each age group the female has a higher count than the male.

Relative Adrenal Weight

In view of the known effect of adrenal cortical hormone on the number of eosinophils, the possibility is examined that the difference in number of eosinophils might be related to a sex difference in adrenal size. The relative adrenal weights of males and females, compared in Table 4, show no significant sex difference in either the normal or hypophysectomized animal. The slight (but not statistically significant) difference in the relative adrenal weight of the 17 hypophysectomized females and 24 hypophysectomized males is due to the difference in the mean times after operation. When males and females are paired, in a group of 12 hypophysectomized the same lengths of time, even this small difference disappears.

In Table 5, the comparison of normal animals is extended for various

TABLE 4. RELATIVE ADRENAL WEIGHT

	Males		Females	
	No. of animals	Mg./100 gm. body wt ±S.E.	No. of animals	Mg./100 gm. body wt. ±S.E.
Normal	37	53.9 ± 2.61	35	56.3 ± 3.53
Hypophysectomized	24	27.5 ± 1.40*	17	32.0 ± 2.22†
	12	32.3 ± 1.78‡	12	31.9 ± 2.13‡

* Time after hypophysectomy. Mean 97.1 (30–180) days.
† Time after hypophysectomy. Mean 66.3 (30–180) days.
‡ Paired for same time after hypophysectomy. Mean 85.8 (30–180) days.

TABLE 5. RELATION OF RELATIVE ADRENAL WEIGHT TO BODY WEIGHT (AGE)

Body weight, gms. (approximate age)	Males		Females	
	No. of animals	Relative adrenal wt. ±S.E. Mg./100 gm. body wt.	No. of animals	Relative adrenal wt. ±S.E. mg./100 gm. body wt.
301–400 (2 months)	7	44.9 ± 6.54	9	55.3 ± 4.66
401–500 (3 months)	16	54.0 ± 3.12	15	55.7 ± 2.29
501–600 (4 months)	14	57.5 ± 3.11	10	52.0 ± 4.84

body weights (ages). The adrenal weights of males and females 2, 3 and 4 months old do not differ significantly. These findings agree with those of Mixner, Bergman and Turner (1943) who reported no discernible difference in the absolute adrenal weights of male and female guinea pigs at various body weights.

DISCUSSION

Reports in the literature on the absolute number of circulating eosinophils in the guinea pig vary considerably. In a summary of the literature to date, Gardner (1947) reported a mean based on males and females of 260 cells/mm.^{3}, Samter, Erickson and Kofoed (1952) found an average of 103 cells/mm.^{3} in a mixed group of 12, and Vaughn (1953) reported an average of 100 cells/mm.^{3} in 5 animals of undesignated sex. Dworetsky, Code and Higgins (1950) found 39 cells/mm.^{3} in the male compared with 105 in the female, whereas Dews and Code (1951) reported only 32 cells/mm.^{3} in the male compared with 56 in the female. Piliero and Gordon (1954), using cardiac blood from anaesthetized guinea pigs, found a mean of 32 cells/mm.^{3} in the male. In an earlier paper (Schweizer, 1953) it was pointed out that handling the guinea pig in the process of drawing the blood sample acts as a mild stress and causes a fall (-29.1%, $P < .05$) in the number of eosinophils within the first hour. This fall was found as early as one half hour after drawing the first sample (unpublished data) and may occur even earlier. The low counts reported by some workers may be due to this mild stress reaction, especially if any appreciable time elapses between removing the animal from its cage and drawing the blood sample.

The reports by Dworetsky, Code and Higgins (1950), and Dews and Code (1951), of a sex difference in the number of eosinophils in the normal guinea pig has been confirmed. The data reported here show that this difference is not related to any sex difference in size of the adrenal gland. The facts reported above, that this sex difference in eosinophils disappears after hypophysectomy, and that males show a greater increase in these cells after hypophysectomy than females, suggest that the level of eosinophils

in the normal animal may be affected by both adrenal cortical and sex hormones. The latter influence is either lacking in the female, or is present in both sexes, but more pronounced in the male. The report of McDermott, Fry, Brobeck and Long (1950) that the eosinopenic effect of epinephrine in adrenalectomized male rats disappeared in the adrenalectomized male castrate was interpreted by them as possibly indicating "a slight overlap in the effect of adrenal and gonadal steroids." It should be noted, however, that Speirs and Meyer (1949) found an increase in eosinophils after administering testosterone propionate to adrenalectomized mice. It is a question if this can be considered comparable to the physiological effects of male sex hormone.

Reports of the influence of hypophysectomy on the number of circulating eosinophils in various species are not uniform. The rise in eosinophils reported here for the guinea pig, is also found in the dog (Simms, Pfeiffenberger and Heinbecker, 1951; Gordon, 1954), and calculations of the absolute number of eosinophils from Crafts' data (1941, 1946) show an increase in the hypophysectomized rat for both sexes, but a greater rise in the male. On the other hand, Asling, Reinhardt and Li (1951) reported a fall in eosinophils in hypophysectomized rats, and Speirs and Meyer (1949) found no significant difference from normal in hypophysectomized male mice.

Speirs (1952) has found an increase in eosinophils with age in the mouse, and other reports (see Vaughn, 1953; Samter 1953) suggest that the older guinea pig also has a higher count (generally attributed to parasitic infestation in older animals). This has not been confirmed in the present report for the age group studied (approximately 2–4 months).

The fall in mononuclear cells reported here for the hypophysectomized male guinea pig is in agreement with that reported by Gordon (1954) for the hypophysectomized male rat. Crafts, however, found an increase in mononuclear cells in both male (1946) and female (1941) rats after pituitary removal.

SUMMARY

Reports of a larger number of circulating eosinophils in the normal female guinea pig than in the male have been confirmed. This difference is not related to size of the adrenal gland which is the same in both sexes. After hypophysectomy, a greater rise in eosinophils in the male than in the female equalizes the count in the sexes. The number of mononuclear cells is the same in normal males and females, but after hypophysectomy a fall in these cells only in the male results in a higher count for the female. There is no sex difference in the number of polymorphonuclear cells in either the normal or hypophysectomized guinea pig.

Within the age range studied (approximately 2 to 4 months old), there was no increase in number of eosinophils with age.

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APPARENT SECRETION OF PROGESTERONE BY HUMAN AND GOAT FETUSES[1]

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IF, AS is usually conceded, the placenta secretes progesterone, one may ask how much of the hormone reaches the fetus. The following experiments were undertaken to study this problem; the data which resulted partially answered the question, but also disclosed an unexpected and interesting fetal-placental relationship. A preliminary report has appeared (Forbes, 1954a).

Ober, Klein, and Weber have recently (1954) presented evidence that the sensitivity of the progesterone assay described by Hooker and Forbes (1947) may be increased up to five-fold in the presence of progesterone:estradiol mixtures in the ratio of 500:1. Little is known regarding circulating levels of endogenous estrogens; it is possible that they may occur in concentrations which would disturb the accuracy of the bio-assay. The writer proposes, therefore, when using this assay to express his results in terms of *progesterone equivalents* (PE). One progesterone equivalent is defined as equal to the *activity* of 1 μg. of progesterone when the latter is assayed by the procedure of Hooker and Forbes in the absence of estrogen and other interfering factors.

METHODS

The plan of the present investigation was to compare progesterone levels in blood from the umbilical artery and vein. Human samples were obtained in the delivery room by drawing about 1 cc. of blood with a hypodermic syringe from the umbilical artery and a similar amount from the umbilical vein, just after delivery of the child but before separation of the placenta. Most of the infants were born spontaneously, but four were delivered near term by Caesarian section. All babies were normal. The goat blood samples, obtained during the latter part of pregnancy[2] also were of 1 cc. and were drawn prior to separation of the placenta.[3] All blood specimens were promptly discharged into vials containing 0.05 cc. of 20% sodium citrate as an anticoagulant. The vials and their contents were then refrigerated until there was an opportunity to separate the plasma

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[2] Gestation in the goat requires 146–151 days (Asdell, 1946).

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TABLE 1. PROGESTERONE EQUIVALENTS/CC. PLASMA FROM HUMAN UMBILICAL BLOOD

	"Free" fraction		"Bound" fraction			"Free" fraction		"Bound" fraction	
	Artery	Vein	Artery	Vein		Artery	Vein	Artery	Vein
C ♂	7.5	1.9			S ♀	2.4	2.2		
S ♀	5.0	<0.4			S ♀	2.3	2.3	<0.6	<0.4
C ♂	4.6	0.25	<0.5	0.6	S ♂	<1.0	0.9	0.5	<0.3
S ♂	4.4	<0.3	<0.7	<0.3	S ♂	0.6	0.5	<0.3	<0.25
S ♀	3.9	1.7	<1.8	0.6	S ♂	<0.6	<1.0	<0.6	<1.0
S ♀	3.5	1.25			S ♂	<0.5	<0.5		
S ♀	3.0	<0.7			S ♀	<0.5	<0.4		
S ♀	2.8	<0.2			C ♀	0.3	<0.25	<0.3	<0.25
S ♂	2.7	1.0							
S ♂	2.7	<0.3	<0.3	0.3					
S ♀	2.0	0.7							
S ♀	1.8	<0.4	<0.3	<0.4					
S ♂	1.5	<0.5	<0.3	<0.25					
S ♂	1.25	0.4	0.4	<0.4					
C ♀	1.1	0.3							
S ♂	0.75	<0.3	<0.25	<0.3	C ♂	<0.4	5.0	<0.4	<0.6

C, delivery by Caesarian section; *S*, spontaneous delivery.

by centrifugation. The plasma was extracted to secure the "free" progesterone. The protein residue was hydrolyzed and extracted to obtain the "bound" progesterone. The extracts were assayed separately in mice of the CHI strain according to procedures already described (Hooker and Forbes, 1947, 1949a). "Free" PE levels were determined for 25 pairs of human and 5 pairs of goat plasma samples; "bound" PE concentrations were established from most of the specimens (Tables 1, 2).

RESULTS AND DISCUSSION

The assays in some cases indicated only that less than a certain amount of hormone was present; the assay does not establish the absence of the hormone. If the assay was negative, the least amount of hormone which could be shown not to be present depended on the size of the plasma sample.

It is apparent that in 16 of the 25 human pairs of samples, and in three of the five caprine pairs, the arterial level of "free" progesterone as measured in PE is appreciably higher than the venous level—i.e., more progesterone is moving from the fetus to the placenta than from the placenta to the fetus. In the case of 8 human and one caprine pairs there is no evidence that arterial and venous levels differ significantly. There is a single instance among the human samples and one among the goat samples in

TABLE 2. PROGESTERONE EQUIVALENTS/CC. PLASMA FROM GOAT UMBILICAL BLOOD

Day of Pregnancy	"Free" fraction		"Bound" fraction	
	Artery	Vein	Artery	Vein
116	4.6	1.0	0.6	<0.3
121	1.6	0.7	<0.2	<0.2
130	<0.2	0.75	<0.2	<0.25
133	<0.2	<0.3	<0.2	<0.3
134	2.8	<0.25	0.2	

which the venous level of the hormone is higher than the corresponding arterial level. The large variations in the concentrations of progesterone in the human specimens do not appear to be correlated with either the sex of the fetus or the nature of the delivery.

The level of PE in the "bound" fractions was always modest and was often too slight to be detected.

It must be emphasized, of course, first that the data for the goat are too limited to support more than the statement that fetal production of progesterone may occur in this species and second that the more extensive data from human blood specimens were obtained only at or close to the end of pregnancy. Even here, fetal production of the hormone was not demonstrated in 9 of 25 cases. Whether fetal release of progesterone is intermittent, whether all fetuses near term can produce the hormone, and whether the phenomenon occurs at earlier stages of human pregnancy requires investigation.

The data pose other questions. Is the compound measured by the assay truly progesterone? When progesterone is being produced by the fetus, what fetal tissue or organ is responsible? How does the placenta return to the fetus less progesterone than it (the placenta) receives?

There may well exist some naturally occurring compound which can duplicate the positive response to progesterone on which the assay is based, even though the extensive specificity tests already conducted have on the whole been satisfactory when mice of the CHI strain are used (Hooker and Forbes, 1949b; Forbes, 1954a). In view of arterial levels which may exceed venous levels by 15-fold or more, it does not appear that the difference is due solely to the possible influence of estrogen on the sensitivity of the assay. Zander and Münstermann (1945) have employed a physico-chemical assay method to determine progesterone levels in blood samples drawn from the human umbilical vein at term. Samples from 4 cases showed an average of 0.34 μg. progesterone/cc. blood. Samples from 3 other cases contained an average of 0.53 μg./cc. plasma. These results are closely comparable to those reported for venous plasma samples in Table 1.

As early as 1905, Starling suggested that one source for "the chemical stimulus or hormone for the growth of the mammary gland" may be "either in the foetus or in the foetal part of the placenta" (page 481). He reported that "extracts" of the viscera of large numbers of rabbit fetuses when injected into virgin rabbits caused mammary growth and lactation. It may be that the fetal gonads secrete progesterone. A seemingly more likely source of this hormone is the adrenal cortex. Production of progesterone or of progestin by the adrenal cortex of the adult has repeatedly been suggested (Engelhart, 1930; Callow and Parkes, 1936; Beall and Reichstein, 1938; Hoffmann, 1940; Lyons *et al.*, 1953; Lazo-Wasem *et al.*, 1954). Hoffmann (1947) has offered interesting evidence for production of the hormone by the adrenal cortex of the human fetus; progesterone secre-

tion by this tissue, he says, develops progressively during the last months of pregnancy, when the output of placental progesterone is diminishing. *Hooker-Forbes-Aktivität* has been detected in extracts of adrenal cortical tissue from human fetuses near term (Ober; Zander and Solth, 1953). Specificity tests (Hooker and Forbes, 1949b; Forbes, 1954b) have shown that when CHI mice are used the bio-assay distinguishes satisfactorily between progesterone and oily adrenal cortical extract, aqueous adrenal cortical extract, desoxycorticosterone, desoxycorticosterone acetate, cortisone acetate, 17-hydroxycorticosterone, and numerous other compounds. Thus it seems unlikely that some of the material assayed as progesterone in the present experiment was instead a related steroid of adrenal cortical origin.

The data here reported indicate that in the majority of cases the "free" progesterone concentration of cord blood is lowered as a result of placental passage. There is no evidence that this reduction is accomplished by "binding" the hormone to protein. Some of the progesterone possibly is converted in the placenta to pregnanediol and other metabolites, but other methods are required to investigate this problem. An alternative hypothesis is that some progesterone moves from the fetal circulation, through the placenta, and into the maternal circulation. Such a concept is not inconsistent with the firm evidence that progesterone, at least in some species, is secreted by the placenta; rather, the possibility arises that not all the progesterone found in the placenta or released by it into the maternal circulation is actually synthesized by that organ.

SUMMARY

Blood samples were drawn from umbilical arteries and veins in 25 human pregnancies at or near term and in 3 goat pregnancies between the 116th and 134th day. The plasmas were bio-assayed for progesterone equivalents. In 16 of the human pairs and in 3 of the caprine pairs the progesterone equivalent level was appreciably higher in the plasma from the umbilical artery than in that from the corresponding umbilical vein. This is interpreted as indicating production of progesterone by the fetus and removal of the hormone from the umbilical blood by the placenta.

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THE REORGANIZATION OF THE NEUROHYPOPHYSEAL STALK FOLLOWING HYPOPHYSECTOMY IN THE RAT[1]

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SINCE Hild and Zetler (1953a) demonstrated that the pharmacodynamic factors present in the posterior lobe of the pituitary gland are intimately bound to a stainable carrier substance, the concept of neurosecretion has been broadened to include both the hormones and the neurosecretory material. More recently, Barrnett (1954) has shown that the active principle of the posterior lobe hormones contains disulfide groups which can be demonstrated histochemically. The presence of these disulfide groups correlates well with the amount of stainable neurosecretory material in the neural lobe under various experimental conditions. Thus, a reduction in hormone content of the neurohypophysis is represented by a decrease of both the microscopically visible amount of neurosecretory granules and the histochemically demonstrable disulfide groups.

Various types of stimuli are capable of causing depletion of the neurosecretory substance in the neurohypophysis. For instance, the perivascular nerve endings of the tractus supraoptico-hypophyseus in the neural lobe of the hypophysis of severely dehydrated animals are devoid of neurosecretory granules (Ortmann, 1950, 1951; Hild, 1951; Hild and Zetler, 1953b; Leveque and Scharrer, 1953). Similarly, the content of antidiuretic hormone of the neurohypophysis of dehydrated animals is low in comparison with that of animals in water balance (Kovács and Bachrach, 1951; Hild and Zetler, 1952).

It is to be expected that after removal of the neurohypophysis the animal should no longer be capable of storing and releasing posterior lobe hormones, or more specifically antidiuretic hormone (ADH). This is the case, but only for a period of from 5 to 7 days following operation. From that time on, ADH again appears in the blood stream (Lloyd *et al.*, 1954a, Lloyd and Pierog, 1955; Mirsky, Stein and Paulisch, 1954).

The question arises as to the source of this antidiuretic hormone. Among

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the possible explanations a "regeneration" of the posterior lobe must be considered in view of the observations of Stutinsky (1951, 1953), who described what seemed to be "regenerated" posterior lobes in several species after hypophysectomy. Following this lead, studies concerning the morphological and functional reorganization of the proximal end of the pituitary stalk of hypophysectomized animals were undertaken. A few of the observations made in the course of this investigation were reported in preliminary form (Leveque, 1954); they are described in detail in this paper. In addition, a variety of experiments were carried out which are designed to test the functional competence of the reorganized posterior lobe. We are interested here only in the question of whether the reorganized neural lobe can be depleted of neurosecretory material, and, thus, be considered as a functional tissue replacing the original neurohypophysis.

MATERIALS AND METHODS

All of the animals used in this investigation were Sprague-Dawley rats of approximately 115–120 gm. obtained within 2 days after hypophysectomy.[3] The rats were maintained on a special diet consisting of bread soaked in water and Kippy dog food on alternate days plus Purina laboratory chow. Oranges, carrots, and lettuce were occasionally added to the diet. Tap water was given *ad libitum*. This diet was adhered to unless otherwise indicated. Except for a few losses during the first two weeks, the animals appeared to thrive under these conditions.

The tissues were fixed in Zenker-formol either by vascular perfusion or by immersion of the material in the fixative. The hypothalamus was removed with the stalk area intact, embedded in paraffin, sectioned sagittally at 7 μ, and stained. The chrome-alum hematoxylin method (Gomori, 1941; Bargmann, 1949) and a modified aldehyde-fuchsin technique (Gomori, 1950; Halmi, 1952) were used for the demonstration of the neurosecretory material.

On the 53 hypophysectomized rats used in this investigation 28 were sacrificed at varying intervals, ranging from 3 days to 5½ months postoperatively. Sagittal sections of the hypothalamus and stalk area were examined for morphological changes following hypophysectomy. These animals served in part as controls for the following experiments.

Sixteen animals which had been hypophysectomized 8 weeks previously were divided into 2 groups. Eight animals were placed on a dry diet of Purina laboratory chow and provided with a drinking fluid consisting of a 2.5% NaCl solution. Eight were fed the same solid diet, but were given no water to drink. In both groups, the rats were near death within 3 to 4 days, at which time they were perfused.

Nine hypophysectomized animals, 4 to 5½ months postoperatively, were placed on 2 mg./100 gm. body weight/day of desoxycorticosterone acetate (DCA).[4] The DCA used was a sesame oil solution given subcutaneously. After 2 weeks of administration of DCA water was withdrawn for 2 days and on the third day 2.5% NaCl solution was given as drinking fluid. The NaCl was continued until the animals were near death (1 to 4 days on NaCl), at which time they were quickly killed with chloroform and the tissues immersed in Zenker-formol. In addition, one animal (R59), in which previous hydration studies had indicated the presence of a remnant of anterior lobe, was dehydrated in the same way but did not receive DCA.

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[3] Hormone Assay Laboratories, Inc., Chicago.

[4] DOCA ACETATE, "Organon," Inc.

OBSERVATIONS

As the hypothalamo-hypophyseal system we consider here the paired supraoptic and paraventricular nuclei, their axons which converge toward the midline and pass through the pituitary stalk, and the neural lobe of the hypophysis. A part of the nerve fibers terminate at the portal vessels of the stalk while the majority end in the vicinity of the posterior lobe vessels (Vazquez-Lopez, 1942; Hagen, 1950; Stotler, 1952).

Morphological Aspects. In Figures 1 and 2 the infundibular area of the normal rat is shown with the comparable area of an animal hypophysectomized for $5\frac{1}{2}$ months. One notices that the proximal end of the pituitary stalk in the operated animal is filled with stainable neurosecretory ma-

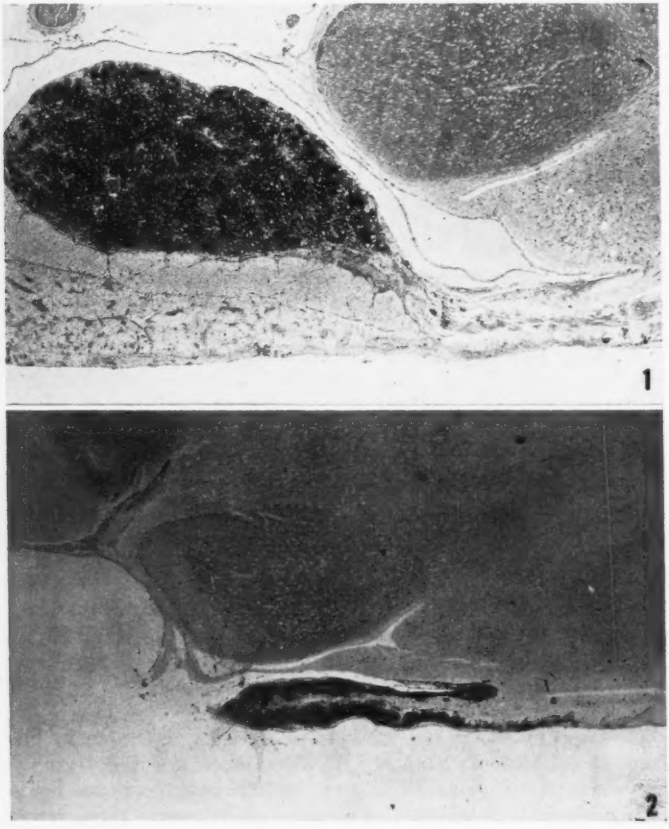

FIG. 1. Pituitary and infundibular process of a normal rat. The posterior lobe is filled with dark staining neurosecretory material. Aldehyde-fuchsin. ×30.

FIG. 2. Median eminence and stalk area of a hypophysectomized rat $5\frac{1}{2}$ months postoperatively. There is an accumulation of stainable neurosecretory material in the median eminence and the pituitary stalk. Technique and magnification as in Figure 1.

terial. This part of the stalk does not contain such large amounts of neurosecretory substance in the normal animal. It seems improbable that remnants of the neural lobe would remain following hypophysectomy owing to the fragility of the stalk at the place where it joins the posterior lobe proper. This becomes evident in gross dissection of the area, for slight tension on the pituitary breaks the stalk at about the same location. Microscopic examination of newly operated animals did not reveal the presence of any remnants of posterior lobe tissue. Since the space normally occupied by the pituitary is empty (Fig. 2), evidently no true regeneration has taken place; it is to be assumed that a reorganization of the proximal end of the pituitary stalk has occurred. Occasionally the reorganized portion is longer; however, in such cases one cannot be certain of the exact point of severance of the stalk.

There is an accumulation of neurosecretory material at the proximal end of the severed pituitary stalk, in the median eminence, and around the stalk vessels in the hypophysectomized animal (Fig. 2). The density of the stainable neurosecretory material is comparable to that found in the normal neural lobe (Fig. 1).

The development of this new storage area for posterior lobe hormones was studied in hypophysectomized rats killed at various intervals following the operation. Three days after hypophysectomy the effects of the traumatic procedure are still evident. Hemorrhage is marked and exudate is present, as shown to the left of the cut surface of the stalk in Figure 3. Various stages of mitosis of the pituicytes are seen at this time but not later. Stainable neurosecretory material is present in the center of the stalk. The material within the nerve fibers causes them to appear quite prominent. This phenomenon is not seen to the same extent in the normal rat.

At five days there is a decided change in the histological picture. The hemorrhage and exudate have almost entirely disappeared and mitotic figures of the pituicytes are absent. A proliferation of endothelial cells occurs and the vascular pattern appears to be in a process of reorganization. The nerve fibers containing a large amount of stainable material are still prominent. In addition, masses of stainable neurosecretory material surround a few blood vessels.

After hypophysectomy of 2 weeks' duration the situation is quite different. The paucity of neurosecretory material is most noticeable (Fig. 4). Only a scattering of microscopically visible granules are evident, and the entire area is exceedingly vascular. The proximal end of the cut stalk appears to be in the process of becoming rounded off.

The decided lack of neurosecretory material can perhaps be explained if one assumes that the vascular pattern has become reorganized to such an extent that once again posterior lobe hormones can be released into the blood stream. It may be that the material is being released at a faster rate

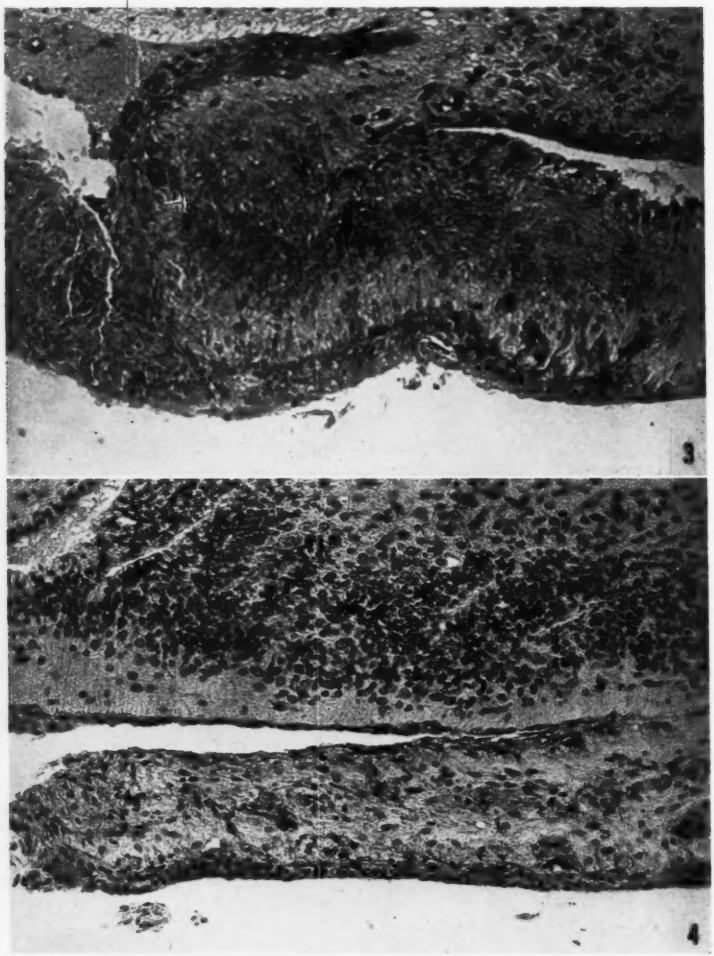

FIG. 3. Cut end of the pituitary stalk in a rat 3 days after hypophysectomy. Chrome hematoxylin phloxin. ×175.

FIG. 4. Cut end of the pituitary stalk 2 weeks after hypophysectomy. Only a few small neurosecretory granules are present. Technique and magnification as in Figure 3.

than the cells of the hypothalamic nuclei can produce the neurosecretory substance. It is conceivable that many of these cells are in the process of recovering from the chromatolytic effects of axon severance, and therefore, are unable to synthesize posterior lobe hormones.

Two months postoperatively there is considerable reaccumulation of stainable neurosecretory substance (Fig. 5). This process starts at about

the third postoperative week and material accumulates steadily until the proximal end of the severed stalk and the median eminence are filled. The neurosecretory substance is particularly dense around the lumina of small vessels. In general, the reorganized end of the stalk has the histological appearance of a normal neural lobe.

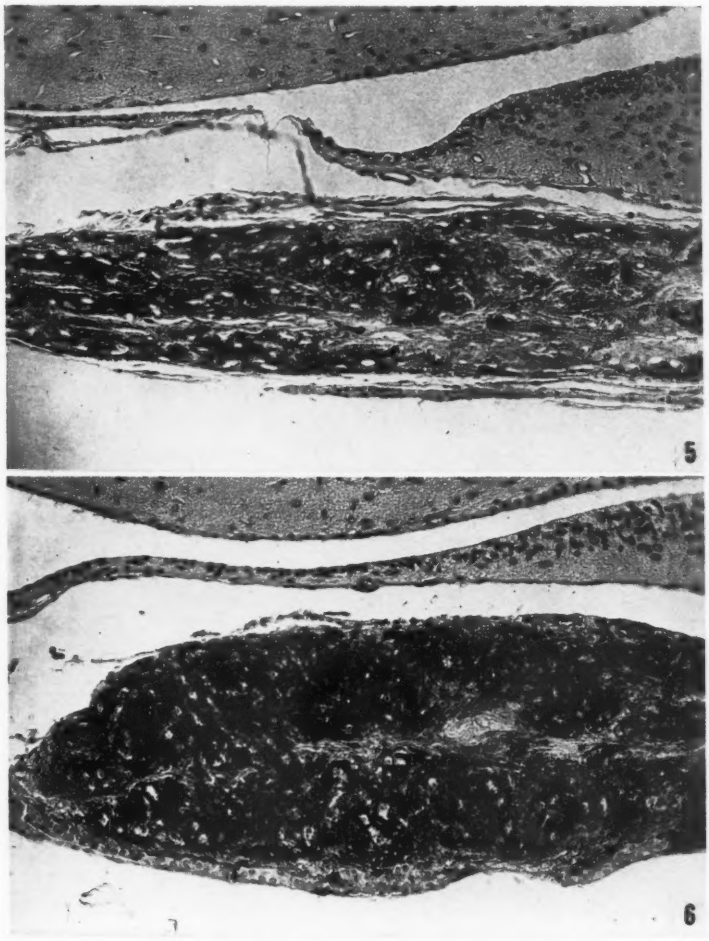

FIG. 5. Cut end of the pituitary stalk in a rat 2 months after hypophysectomy. Reorganization of the stalk with a reaccumulation of stainable neurosecretory material. Chrome hematoxylin phloxin. ×175.

FIG. 6. Severed pituitary stalk in a rat 5½ months after hypophysectomy. An increase in width of the reorganized structure with a dense reaccumulation of stainable neurosecretory material. Technique and magnification as in Figure 5.

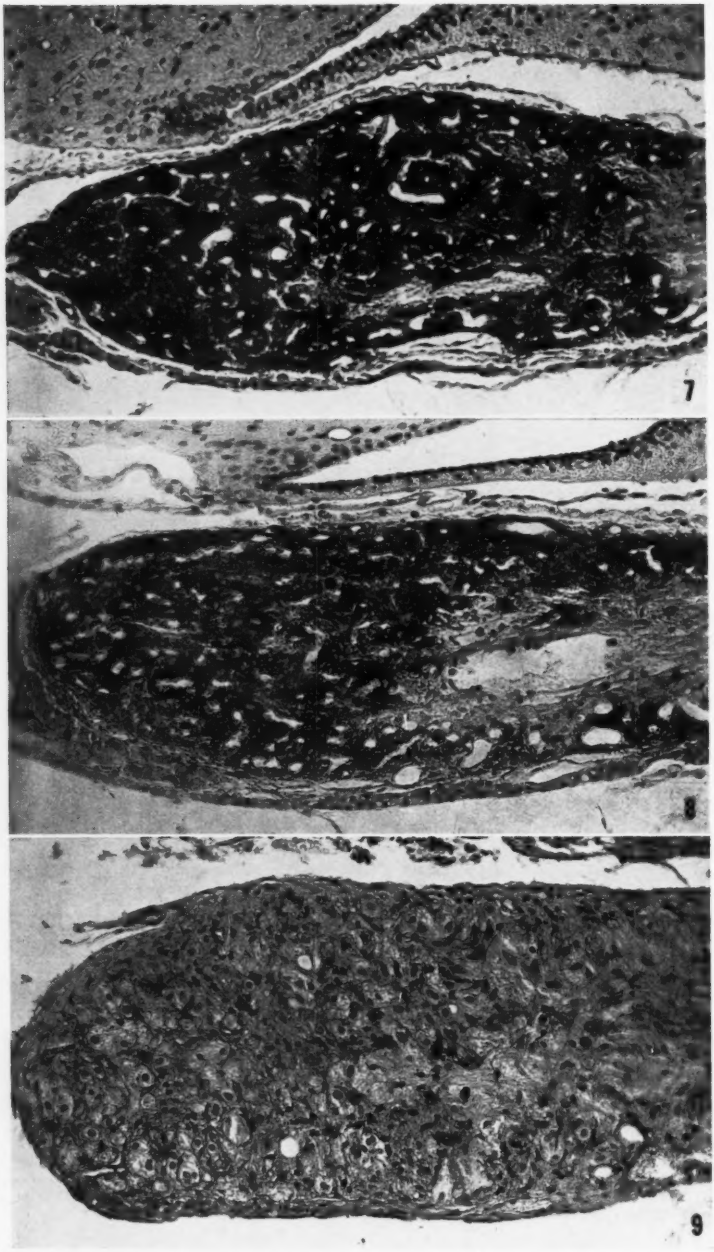

FIG. 7. Reorganized pituitary stalk 2 months after hypophysectomy in an animal thirsted for 4 days. There is no histological evidence of mobilization of neurosecretory

At 5½ months the normal appearance of this structure is intensified. The cut end of the stalk is definitely demarcated and has increased in width (Fig. 6). There can be little doubt that a storage depot for the posterior lobe hormones has been reconstituted. Since it cannot be stated that a true regeneration has occurred, the term "reorganization" is preferred to describe the phenomenon which has taken place at the severed end of the pituitary stalk in the hypophysectomized rat.

Another interesting observation is that of a heavy accumulation of neurosecretory material in the immediate vicinity of the portal vessels to a much greater extent than that found in the unoperated animal. The question arises as to the ultimate course of these vessels in the hypophysectomized animal, since no true portal system can now exist.

Functional Aspects. Since the proximal end of the cut pituitary stalk does reorganize into a posterior lobe-like structure, it was of interest to investigate the possibility that it may also resume functional activity. After allowing 8 weeks for the morphological reorganization to take place, 16 hypophysectomized rats were subjected to dehydration. Eight of the animals were given 2.5% NaCl as drinking fluid and 8 other rats were kept entirely without water as long as they would survive (3 to 4 days in either case). Following the period of dehydration, the content of neurosecretory material in the reorganized posterior lobe was found to be the same as in the non-dehydrated animal; possibly even slightly increased (compare Figs. 5 and 7). The neurosecretory material was apparently not mobilized in any great quantity, and no depletion resulted (Figs. 7 and 8).

In view of the unsuccessful attempts to mobilize the neurosecretory material in the reorganized neurohypophysis of the operated animal by measures which cause depletion of the posterior lobe in the normal rat, a different approach to the problem was undertaken. Since adrenal cortical substitution therapy causes an acceleration of water exchange in the hypophysectomized rat (Boss, Osborn, and Renzi, 1952), it was reasoned that perhaps by increasing the rate of water turnover within the operated animal a greater demand for antidiuretic hormone could be produced. Thus, a depletion of neurosecretory material may occur within the survival time of the dehydrated animal. Nine hypophysectomized rats, 5½ months postoperatively, were injected with desoxycorticosterone acetate (DCA)

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material from the reorganized tissue. Chrome hematoxylin phloxin. ×175.

FIG. 8. Reorganized stalk 2 months after hypophysectomy in a rat given 2.5% NaCl as drinking fluid for 4 days. No depletion of neurosecretory material can be detected. Technique and magnification as in Figure 7.

FIG. 9. Reorganized pituitary stalk 5½ months after hypophysectomy in a rat given DCA for 19 days and dehydrated for the last 6 days. The depletion of neurosecretory is almost complete. Technique and magnification as in Figure 7.

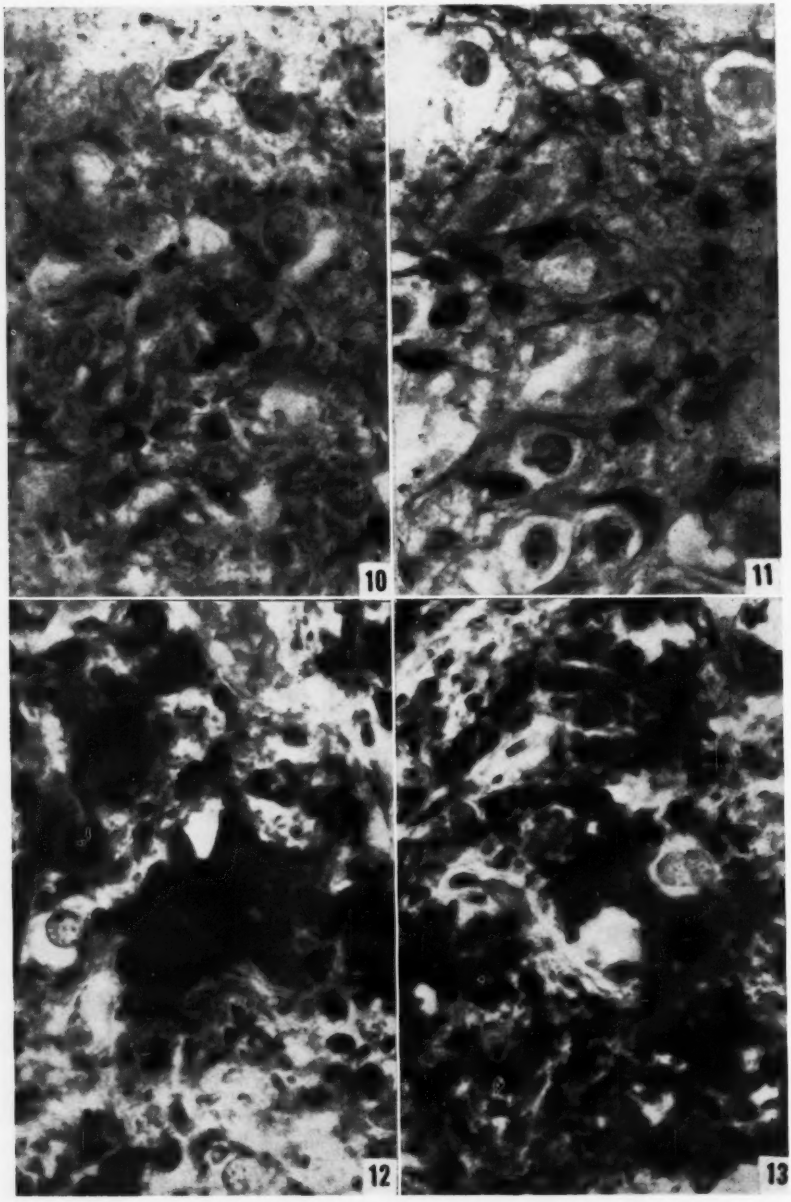

Fig. 10. Posterior lobe of a normal rat in an extreme degree of depletion of stainable neurosecretory material. Chrome hematoxylin phloxin. ×1000.

Fig. 11. Reorganized pituitary stalk devoid of neurosecretory granules of a rat hypophysectomized for 5½ months. The histology is strikingly similar to that of a depleted normal posterior lobe. Technique and magnification as in Figure 10.

for two weeks prior to dehydration. Then water was withdrawn for two days and on the third day 2.5% NaCl was given as drinking fluid until the animals were near death (1 to 4 days on NaCl). During this time the administration of DCA was continued until the termination of the experiment.

Histological examination of the reorganized neurohypophysis in these animals showed almost total depletion of neurosecretory granules. The depletion was equivalent to that found in the posterior lobe of the normal rat after 10 to 13 days of dehydration (Leveque and Scharrer, 1953). In some cases the survival time was two days longer than in any of the dehydrated animals in the series not given DCA, but in a few cases the survival time was less. Regardless, all of these animals showed extreme depletion (Fig. 9).

Since the reorganized posterior lobe of the hypophysectomized rat could be emptied of its neurosecretory material, it was possible to examine the histological picture in greater detail. The size, shape, and location of the pituicytes, as well as the number per unit volume of tissue, is the same in both the normal posterior lobe and the reorganized structure in the hypophysectomized animal (Figs. 10 and 11). Also, the organization of the capillary bed appears identical. Illustrations of the normal and reorganized neurohypophysis filled with neurosecretory granules are included for purposes of comparison (Figs. 12 and 13). The size, shape, and intensity of staining of the neurosecretory granules appears the same. Thus, one finds little, if any, difference in the histology of the normal neurohypophysis and the reorganized posterior lobe tissue of the hypophysectomized rat by the methods used in this investigation.

It should be mentioned that in tissue immersed in the fixative many of the pituicytes appear greatly enlarged and possess a clear cytoplasm. This phenomenon does not occur to any great extent in material fixed by vascular perfusion. Thus, the enlargement of pituicytes seen in some of our material (Fig. 11) may be attributed to the method of fixation (Scharrer, 1938) rather than to actual cell changes occurring in the process of reorganization.

DISCUSSION

The observation in the hypophysectomized rat that the proximal end of the pituitary stalk reorganizes into a tissue structurally resembling a

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FIG. 12. Posterior lobe of a normal rat, filled with neurosecretory granules. Technique and magnification as in Figure 10.

FIG. 13. Reorganized pituitary stalk filled with neurosecretory material in a rat hypophysectomized for 5½ months. The size, shape and distribution of neurosecretory granules is indistinguishable from that found in the normal posterior lobe. Technique and magnification as in Figure 10.

normal neural lobe definitely confirms Stutinsky's (1951) findings. In addition, the demonstration that the reorganized neurohypophysis is functionally competent in that it is capable of releasing neurosecretory material should be of considerable interest. This phenomenon not only explains some of the unresolved aspects in the study of the effects of hypophysectomy, but, at the same time, it points to a number of new problems.

Among the latter is the question of how the nerve fibers regenerate after severance. Tello (1912) has shown that new terminals do develop. However, the establishment of new relationships between nerve endings and blood vessels after hypophysectomy should be explored in detail. In particular, the question arises as to the nature of the vessels with which these nerve endings become associated and into which the neurosecretory material is released. Evidently these vessels belong to the primary plexus of the hypophyseal portal system that serves as a vascular link between the median eminence and the anterior lobe. Presumably they establish connections with systemic vessels through which the neurosecretory material is carried into the general circulation. A study of the process of reorganization of the vascular pattern of the pituitary stalk following hypophysectomy is under way at the present time.

The observation of an accumulation of stainable neurosecretory material in the vicinity of the portal vessels and particularly in the region of the median eminence, first reported by Stutinsky (1951) and described in some detail in this paper, may well explain Sato's (1928) unresolved findings. Sato reported that extracts from the tissue of the tuber cinereum showed much greater antidiuretic activity after hypophysectomy than did similar extracts from the normal animal. The morphological evidence given in our paper shows that there should be considerably more posterior lobe hormone in this area in the hypophysectomized animal than in the comparable area of the normal rat (compare the amounts of neurosecretory material in the stalk and the median eminence in Figures 1 and 2). Barrnett (1954) reports a greater than normal quantity of histochemically demonstrable disulfide groups in the infundibular stalk in the hypophysectomized rat. Also, Lloyd and Pierog (1955) find an increase in ADH content in the region of the median eminence following hypophysectomy.

The histological demonstration of an apparently functional tissue replacing the neurohypophysis after hypophysectomy is significant in the light of the evidence presented by other investigators who have used bioassay methods. Assays for ADH have shown that it reappears in the blood following the removal of the hypophysis (Mirsky, Stein, and Palisch, 1954; Lloyd *et al.*, 1954; Lloyd and Pierog, 1955). Maren and Bodian (1951) produced antidiuresis in the hypophysectomized animal by drugs which are believed to cause release of ADH via the neurohypophysis. They did not interpret their results as an indication of a reorganization of the pitui-

tary stalk into a functional unit, but attributed them to remnants of neural lobe tissue that had remained in situ. It is more probable that Maren and Bodian were dealing with stalk tissue reorganization rather than with remnants of the original neural lobe.

In blood assay studies of hypophysectomized animals, sources of antidiuretic substances other than the hypothalamo-hypophyseal system should be considered. However, the available morphological evidence, the histochemical data of Barrnett (1954), and the physiological studies of Lloyd and Pierog, 1955, reported in the paper which follows in this issue, all indicate that the hypothalamo-hypophyseal system is the source of the ADH in the cirdulation of the hypophysectomized rat.

The question of whether the reconstituted neural lobe in the hypophysectomized rat is capable of releasing hormones has its counterpart in invertebrate physiology. After the removal of the crustacean sinus gland, which is comparable to the posterior lobe in vertebrates, a new sinus gland is formed (Passano, 1951; Bliss and Welsh, 1952; Enami, 1954). Eventually the physiological processes disturbed by the extirpation of the sinus gland return to normal (Bliss, 1953). Thus a parallel situation seems to exist in the neurosecretory system of a mammal, such as a rat, and an invertebrate, such as a decapod crustacean.

Certain aspects of the pituitary-adrenal relationships as they pertain to water balance should be considered in view of the evidence that the severed pituitary stalk is capable of assuming the structure and function of neural lobe tissue in the hypophysectomized animal. Following hypophysectomy in the rat there is a disturbance of water balance which manifests itself in a slowness of response to a water load (Joseph *et al.*, 1944) rather than in a marked disturbance of the electrolyte balance. The glomerulosa of the adrenal cortex appears normal in the rat following hypophysectomy (Deane and Greep, 1946), and it has been shown that there is a quantitative rather than a qualitative change in the electrolyte pattern of the hypophysectomized mouse (Jones and Roby, 1954). Since ADH has been detected in the circulation postoperatively (Lloyd *et al.*, 1954; Lloyd and Pierog, 1955; Mirsky, Stein, and Paulisch, 1954), we may assume that a physiological relationship again exists between the reorganized neurohypophysis and the partially atrophic adrenal cortex in the hypophysectomized animal.

In a companion paper Lloyd and Pierog (1955) report that extracts prepared from the hypothalamic region of the median eminence in the hypophysectomized rat contains less ADH after dehydration than do similar extracts in hydrated animals. In our investigation we probably did not succeed in causing a detectable depletion of neurosecretory material from the reorganized posterior lobe under conditions of dehydration alone because ADH was not mobilized in sufficient quantities to be demonstrated by the histological methods used. However, by giving adrenal cortical

substitution therapy to hypophysectomized animals water metabolism may have been accelerated sufficiently to cause an increased demand for ADH, and, thus, a total depletion of neurosecretory material occurred in the reorganized posterior lobe of the totally hypophysectomized rat.

It can perhaps be stated that a high degree of polydipsia and polyuria does not always develop in neurohypophysectomized animals, provided that the hypothalamic cells which produce the posterior lobe hormones have not been completely destroyed. A new posterior lobe can be reconstituted which is capable of releasing ADH. However, stalk sectioning causes lysis of a considerable number of hypothalamic cells, which results in a diminution of the amount of ADH available to the organism. When the parapharyngeal approach is used and the hypophysis is removed by means of a pipette, the supraoptic nuclei are invariably reduced to one-third of their normal size. It is conceivable that the number of the remaining cells depends upon the level of serverance of the stalk. Perhaps the demand for ADH is often greater than the capacity to produce the hormone. This situation may explain some of the conflicting results concerning the degree of diabetes insipidus in neurohypophysectomized animals.

In the case of total hypophysectomy the preliminary phase of polydipsia and polyuria that usually occurs can be explained on the following basis: while no ADH is available immediately after operation the diuretic principles of the adrenal cortex are still being released, but in gradually diminishing amounts until the output drops to the level of that of the atrophic adrenal gland. During the time that the adrenal cortex is undergoing atrophy the severed stalk of the neurohypophysis is in the process of reorganization. Eventually in these animals a water balance becomes reestablished which is based upon the diuretic principles produced by the surviving adrenal cortex and the antidiuretic action of the posterior lobe hormones released from the reorganized neurohypophyseal stalk. This hypothesis would seem to explain adequately the phenomena observed in the rat, and is essentially in agreement with Lloyd's interpretation. However, it remains to be seen if corresponding considerations are equally applicable to observations made in other species.

SUMMARY

The proximal end of the severed pituitary stalk in the totally hypophysectomized rat was studied from 3 days to $5\frac{1}{2}$ months following the operative procedure. This investigation confirms the observation reported by Stutinsky that the proximal end of the severed stalk becomes reorganized into new neural lobe tissue that is capable of storing neurosecretory material containing the posterior lobe hormones. In addition, the functional competence of the reorganized neurohypophysis was explored. By our methods mobilization of the neurosecretory substance from the reconstituted structure could not be detected when dehydration procedures

were used alone. However, the administration of adrenal cortical substitution therapy plus dehydration caused the reorganized neurohypophysis to be depleted of neurosecretory material. Thus, morphological evidence is presented to the effect that the reorganized neurohypophysis could be a possible source of the antidiuretic substance found in the blood of the hypophysectomized rat by other investigators.

Certain aspects of the mechanism of water balance in the hypophysectomized rat have been discussed, including a possible explanation of the role of the reorganized neural lobe tissue in experimental diabetes insipidus.

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STUDIES OF THE ANTIDIURETIC ACTIVITY OF BLOOD AND HYPOTHALAMUS OF HYPOPHYSECTOMIZED RATS

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IN RECENT years the concept of secretion by specialized nervous tissue has become generally accepted (Scharrer and Scharrer, 1954). One of the more important neurosecretory structures is the hypothalamico-neurohypophyseal tract. Essentially as the result of histological evidence it has been proposed that the antidiuretic hormone of the posterior pituitary is formed in the supraoptic and paraventricular nuclei of the hypothalamus and travels along nerve pathways to be released into the blood stream through the blood vessels of the posterior pituitary.

It has usually been considered that the totally hypophysectomized animal does not release antidiuretic hormone into its blood (Birnie, Jenkins, Eversole and Gaunt, 1949; Ames and van Dyke, 1952; Ginsburg and Heller, 1953). However, in a recent communication from this laboratory it was reported that while the blood of animals which have been hypophysectomized for a short period of time contains no detectable antidiuretic hormone, the blood of animals hypophysectomized for a longer period of time contains appreciable amounts of antidiuretic activity (Lloyd, Loewy, Pierog, Bradwick and Sostheim, 1954a & b). Mirsky, Stein and Paulisch (1954) have also found antidiuretic material in the blood of hypophysectomized animals. These observations are best explained by the hypothesis that the hypothalamus forms and releases antidiuretic hormone after a functional reorganization in the absence of the posterior lobe. This publication extends these findings and also describes measurement of the content of antidiuretic hormone of the hypothalami of hypophysectomized animals under different conditions.

METHODS AND MATERIALS

Assay of antidiuretic activity was performed by the method of Jeffers, Livezey and Austin (1942) utilizing the alcohol anesthetized rat to which the antidiuretic substance was given intravenously. This method permits detection of antidiuretic activity of the order of 15 microunit equivalents of vasopressin. A standard dose of 20 microunits of vasopressin was always given at the beginning and, if possible, at the end of the assay procedure in order to check the sensitivity of the assay animal. Hypophysectomized animals were of the Sprague-Dawley strain, purchased from Hormone Assay Laboratories. All of the experimental animals were maintained on a diet high in protein and calories, composed of horsemeat, cream, vegetable oil, sugar and vitamins blended in a mixer.

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The hypophysectomized animals maintained a good rate of weight gain on this diet.Two groups of hypophysectomized rats were studied. In one group the animals were at least 30 days postoperative. The second group was composed of animals which were about one week postoperative. Careful examination in the region of the sella turcica grossly and histologically failed to demonstrate remnants of the hypophysis. The animals which had been hypophysectomized for 30 days or longer all showed atrophy of the target organs of the anterior pituitary trophic hormones. Animals with diabetes insipidus had the neurohypophysis removed, but had functional anterior pituitary glands. These animals had no atrophy of the target organs of the anterior pituitary gland and had spontaneous water turnover of at least double that of the normal animals. In many instances, water turnover was 10 times normal.

In most of the studies the donor animals were allowed water *ad libitum* but food was withdrawn 18 hours before sacrifice. The dehydrated animals were allowed food but no water for from 24 to 48 hours before sacrifice. The hydrated animals were given a liquid diet consisting of milk and 5% glucose solution for 72 hours before sacrifice. Blood was obtained by cardiac puncture under Nembutal anesthesia and immediately placed in iced centrifuge tubes. Serum was separated and assayed as quickly as possible, usually within ten minutes after collection of the blood. The antidiuretic content of serial dilutions in water of the homogenized block of tissue containing the hypophthalamas was also measured. This block was approximately 5 mm. wide extending from anterior of the optic chiasm to posterior of the mammillary bodies and including the floor of the third ventricle. Several blocks were sectioned parallel to the floor of the third ventricle and the majority of antidiuretic material was found in the portion containing the median eminence. Comparable blocks from other portions of the brain contained no antidiuretic substance. The activity of the posterior pituitary was also assayed in a similar manner.

RESULTS

The number of materials which could be assayed in one day was limited by the duration of diuresis in the test animals and it was frequently not practical to determine the absolute level of assay at which a positive response was found, or the lowest level at which no activity was detected. The absolute level of antidiuretic activity may have been higher than the positive figure recorded, but the degree of antidiuretic response was so low that the level of antidiuretic activity was thought to be not much greater the the level of assay.

Results are summarized in Table 1. The antidiuretic content of sera is plotted in Figure 1. The serum of intact animals generally contained antidiuretic activity in the range of from 100 to 400 microunit equivalents of vasopressin per ml. of serum. The serum of only five of 22 recently hypophysectomized animals had antidiuretic activity. In contrast to this, the serum of 28 of 42 animals which had been hypophysectomized for over one month contained antidiuretic activity of from 100 to 400 microunit equivalents of vasopressin per ml. The serum of the animals with diabetes insipidus did not contain any detectable antidiuretic activity.

The posterior pituitaries of 9 normal animals hydrated *ad libitum* had antidiuretic activity which ranged from approximately 10 to more than 200 milliunit equivalents of vasopressin. The results of measurement of anti-

TABLE 1

Type of donor animal	ADS of SERUM γ unit equivalents of vasopressin	ADS OF PITUITARY γ unit equivalents of vasopressin	ADS OF HYPOTHALAMUS γ unit equivalents of vasopressin
Intact *ad libitum* hydration	+100 to 400 (13)* −100 (4)	10,000 to 200,000 (9)	5,000 (32) 15,000 (2)
Hypophysectomized 7 days postop. *ad libitum* hydration	+100 to 200 (5) −100 (11) − 50 (6)		0.5 (3) 1.25 (1) 10 (2)
Hypophysectomized 30 days postop. *ad libitum* hydration	+100 to 400 (28) −100 (14)		1.00 (1) 5.00 (1) 10.00 (6) 15.00 (1)
hydrated			25.00 (4)
dehydrated			5.00 (2) 2.50 (1) 1.25 (1)
Diabetes Insipitus	−100 (8) +100 (5)		0.50 (1) 2.50 (2) 5.00 (6) 10.00 (1)

* Numbers in parentheses indicate number of animals assayed.

diuretic activity of hypothalami are graphed in Figure 2. Hypothalami of five intact animals hydrated *ad libitum* contained from 5 to 15 milliunit equivalents of vasopressin of antidiuretic activity. Three of the six recently hypophysectomized animals tested had less than 0.5 milliunit equivalents of antidiuretic activity in the hypothalami. In contrast, the hypothalami of 9 animals hypophysectomized for more than 30 days contained essentially the same general range of antidiuretic activity as was found in the intact animals. Upon hydration, the antidiuretic activity was increased to 25 milliunits and dehydration produced a considerable decrease in antidiuretic activity. Hypothalami of 10 animals with diabetes insipidus had what probably was a lower range of activity than was found in the intact normal animal or in animals which had been hypophysectomized for the same length of time. They did not contain nearly as much activity as was found in hypothalami of hypophysectomized animals which had been hydrated with an amount of fluid comparable to that consumed by the animals which diabetes insipidus. The level was more nearly comparable to the level found in hypophysectomized animals which had been dehydrated.

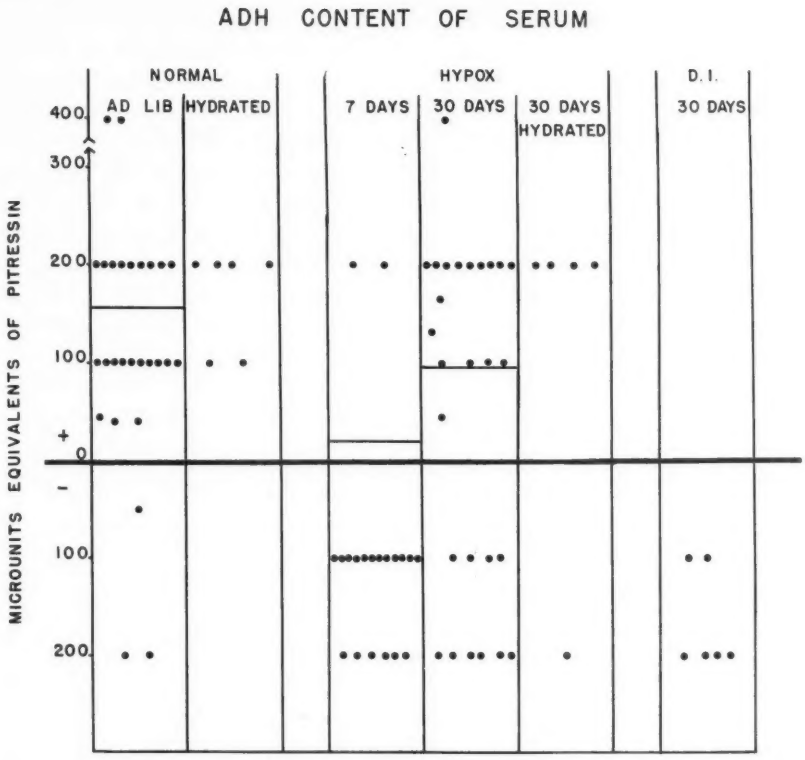

FIG. 1. Antidiuretic content of serum: Each point above the baseline indicates a serum giving a positive response at the assay level tested. The points below the baseline indicate sera which gave a negative response at the assay level tested.

The levels of antidiuretic activity in blood, hypothalami and posterior lobe are summarized in Figure 3.

DISCUSSION

Experiments on the control of hypothalamic and posterior pituitary secretion in intact animals will be described in a subsequent paper.

As has been previously reported (Lloyd, Loewy, Pierog, Bradwick and Sostheim, 1954a), the serum of many animals which have been hypophysectomized for some time contains appreciable antidiuretic material. Mirsky, Stein and Paulisch (1954) have found an increase in antidiuretic activity of blood of hypophysectomized animals following a noxious stimulus. It seems probable that a reorganization permitting release of antidiuretic hormone into the circulation has taken place within the tissues remaining following removal of the neurohypophysis. It would appear from this study that immediately after hypophysectomy the hypothalamus

is depleted of antidiuretic hormone. This may be the result of the well-known release of ADH under stress (Verney, 1947; Kelsall, 1949; Mirsky, Stein and Paulisch, 1954). During the first few days after hypophysectomy the circulating level of antidiuretic hormone is low. The level of hormones of anterior piutitary origin which further water excretion may not decline as rapidly. The effects of adrenocortical and thyroid hormones would probably decrease relatively more slowly than those of the antidiuretic hormone, which is inactivated rapidly and which has a duration of action of only a few minutes. This would result in a relative preponderance of diuretic factors, causing a polyuria and polydipsia. In addition, immediately after hypophysectomy the animal would still have the preoperative solute levels which would also contribute to a relatively large water turnover.

The low level of antidiuretic activity of the hypothalamus present for several days after operation could be the result of a local reflex, which causes a temporary block in formation of the neurosecretory material.

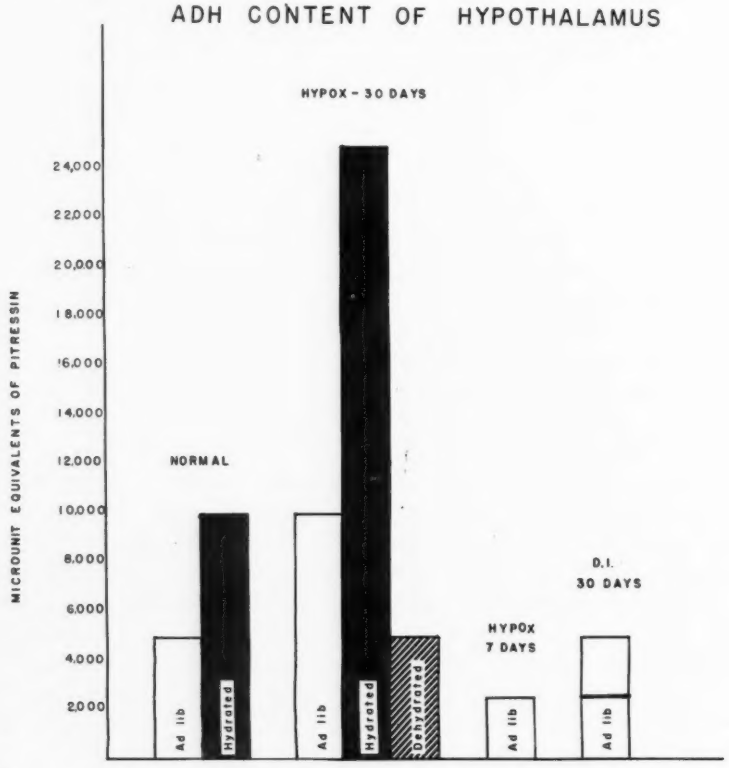

FIG. 2. Antidiuretic content of the hypothalamus.

After an interval of several weeks or more, the hypothalamus appears to regain its capacity to form the antidiuretic hormone and, in addition, a reorganization takes place which permits the release of the hormone into the blood stream. Billenstien and Leveque (1955) who have studied by histological means the neurosecretory material present in the median eminence, have observed comparable events. They find that soon after hypophysectomy the neurosecretory substance has a very low concentration. As the hypophysectomized animal acquires new homeostatic mechanisms, there begins to appear a much larger concentration of antidiuretic hormone in the hypothalamus. This appears to be associated with a reorganization of the median eminence which permits transfer of substances to blood.

The demonstration the ability of the median eminence to secrete antidiuretic hormone confirms and explains the observations of Sato (1948), which until now have seemed paradoxical. Sato reported an in-

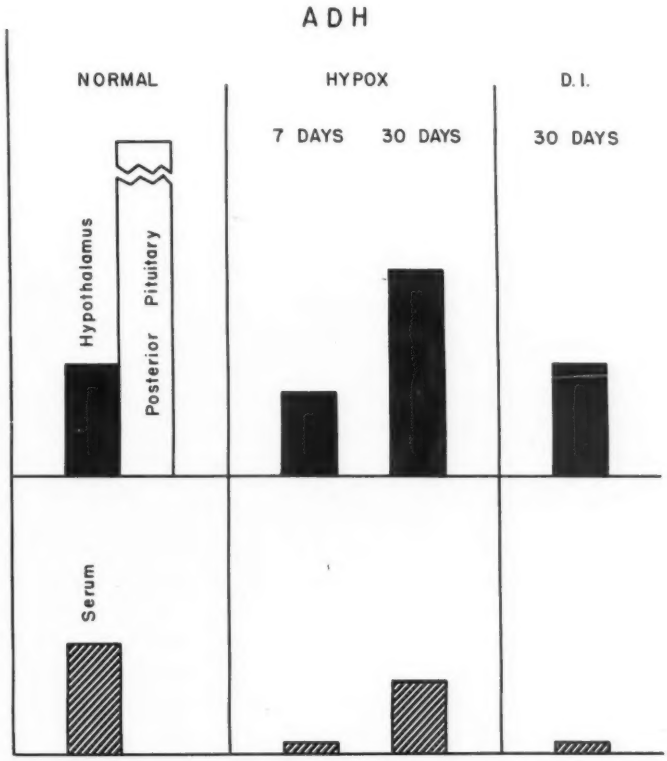

FIG. 3. Schematic representation of the relationships between antidiuretic content of serum, hypothalamus and posterior pituitary gland.

crease in antidiuretic activity of the median eminence following hypophysectomy. Melville and Hare (1945), on the other hand, have reported a decrease in antidiuretic activity of the portions of the hypothalamus which contain the supraoptic and paraventricular nuclei. A few experiments carried out in this laboratory confirm the finding that the greatest portion of antidiuretic activity lies in the area containing the median eminence. The studies of Billenstien and Leveque (1955) indicate that it is in the median eminence that the greatest concentration of neurosecretory material is found following reorganization of the hypothalamus and suggest that from this site antidiuretic hormone can be released.

The most reasonable explanation of the finding of low levels of antidiuretic material in the hypothalami of animals with diabetes insipidus is that the anterior lobe utilizes the antidiuretic material, thereby depleting the hypothalamus. There are three other possibilities which do not seem well-supported by the evidence presently available. These are: The hypothalamus contains little antidiuretic substance because it is depleted in response to dehydration. It is true that the animal with diabetes insipidus excretes more water than it is able to take in and usually is in a state of mild chronic dehydration. However, if the animal were secreting large amounts of antidiuretic hormone and thereby depleting the hypothalamus, it is not reasonable to expect that it would continue to have diabetes insipidus. Instead the antidiuretic hormone should cause conservation of water and amelioration of the polyuria. This does not occur. In addition, low levels of antidiuretic activity in blood were found in these animals. Therefore, it seems unlikely that the low level of antidiuretic hormone found in the hypothalamus is the result of exceptionally rapid secretion of hormone into the systemic circulation.

Another possibility is that the rate of secretion is high, but that for some reason there is an increased rate of inactivation of the hormone which prevents the blood levels from ever becoming sufficiently high to produce an effect on the kidney. This hypothesis would require the supposition that in the animal with diabetes insipidus inactivation of antidiuretic hormone proceeds at a rate greater than in intact or hypophysectomized animals. As first pointed out by Heller and Urban (1935) and since confirmed by others (reviewed by Lloyd and Lobotsky, 1954), the principal site of inactivation of antidiuretic hormone is in the liver. However, attempts at increasing the rate of inactivation of vasopressin by liver have not been successful (Lloyd, 1955). The authors are unaware of any studies of the rate of inactivation of vasopressin by animals with diabetes insipidus but no evidence exists suggesting that the rate of inactivation is increased in these animals.

A third possibility is that the anterior lobe in some way depresses formation of antidiuretic material by the hypothalamus. This would presuppose

an unproven retrograde flow through the hypophyseal portal vessels for which no evidence exists.

The low level of antidiuretic hormone in the hypothalamus appears most likely to be the result of utilization of the antidiuretic substance by the anterior lobe. On the basis of anatomical findings Rothballer (1953) and Scharrer and Scharrer (1954) have suggested the possibility that the neurosecretory material of the supraoptico-neurohypophyseal system might be the neurohumoral substance responsible for release of ACTH from the anterior lobe. The release of antidiuretic material following various stresses (Verney, 1947; Kelsall, 1949; Mirsky, Stein and Paulisch, 1954) and the increase in circulating antidiuretic material following adrenalectomy (Birnie, Jenkins, Eversole and Gaunt, 1949; Mirsky, Stein and Paulisch, 1954) have also led Mirsky, Stein and Paulisch to suggest that the antidiuretic hormone might be concerned with ACTH release. Substantial confirmation of this hypothesis has been presented by the demonstration by McCann and Brobeck (1954) and of McCann and Snydor (1954) that lesions in the supraoptico-neurohypophyseal tract produce decreases in adrenal cortical responsiveness and in circulating ACTH levels which are inversely proportional to the severity of the diabetes insipidus. In the light of these reports, a reasonable hypothesis is that the anterior lobe removes antidiuretic material from the hypothalami of the animals with diabetes insipidus while this substance remains in the hypothalami of totally hypophysectomized animals. Experiments designed to offer further light on the mechanisms involved are now in progress.

SUMMARY

Serum of intact rats, fasted but hydrated *ad libitum*, contains antidiuretic activity of between 50 to 400 microunit equivalents of vasopressin per ml. of serum. Serum of recently hypophysectomized rats contains much less antidiuretic activity. Serum of animals hypophysectomized for 30 days or longer contains only slightly less antidiuretic activity than is found in serum of intact animals. Serum of animals with diabetes insipidus contained very little antidiuretic activity.

The posterior pituitaries of intact, fasted but *ad libitum* hydrated rats, contain from 10,000 to greater than 200,000 microunit equivalents of vasopressin.

Hypothalami of recently hypophysectomized animals contain less antidiuretic activity than those of intact animals, values being from 500 to 10,000 microunit equivalents of vasopressin.

Hypothalami of animals hypophysectomized for 30 days and fasted but hydrated *ad libitum* contained essentially the same amount of antidiuretic activity as was present in hypothalami of intact animals. Hydra-

tion increased the antidiuretic content and dehydration decreased it. The hypothalami of animals with diabetes insipidus tended to have somewhat lower levels of antidiuretic activity than were present in animals hypophysectomized for the same length of time.

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PYRUVATE OXIDATION IN TESTICULAR TISSUES DURING FURADROXYL-INDUCED SPERMATOGENIC ARREST[1]

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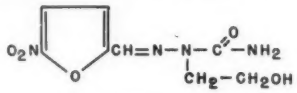
NELSON and Steinberger (1952) noted that the testes of rats receiving the nitrofuran Furadroxyl (Fig. 1) orally were similar histologically to those of patients suffering spermatogenic arrest. The stage of spermatogenic arrest is illustrated in Figure 2, sections (1), the normal picture, and (2) after 30 days on a diet to which Furadroxyl had been added. In the latter tubules it can be noted that mature sperm were not present and that there was no development of any cells beyond the primary spermatocyte stage. The testes were about one-third normal size, but body weight was not affected.

The Pauls and their associates (1952, 1953, 1954) have compiled considerable data on the metabolic aspects of this nitrofuran poisoning of the testis. The important points from their work which have a bearing on the



FURADROXYL

FIG. 1.

studies we are reporting are: (1) Succinate oxidation is apparently not impaired by the nitrofurans, (2) glucose utilization is inhibited during the Furadroxyl feeding period and does not return to normal until approximately 42 days after the nitrofuran is removed from the diet—an event which one of us (W.O.N) has shown coincides with the return to normal of sperm development, and (3) citrate formation from oxalacetate is inhibited by the nitrofurans, apparently at the step in which acetylcoenzyme A is formed from pyruvate. This difficulty is eliminated in 7–8 days after the nitrofuran has been removed from the diet.

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In an attempt to elucidate further the mechanism of the nitrofuran effect on testicular tissues, we have undertaken a number of studies which are described in this report. These studies include (1) observations on a series of biopsied human testicular tissues, (2) observations on a series of rats during the recovery period after 30 days on a Furadroxyl containing diet, (3) attempts to restore the ability of the poisoned tissue to oxidize pyruvate *in vitro*, (4) attempts to hasten the recovery, (5) comparison of Furadroxyl-poisoned testicular tissues with those obtained at various times after hypophysectomy, a procedure which Houssay (1951) states is known to halt sperm production at the secondary spermatocyte stage, and (6) comparison of Furadroxyl-poisoned tissues with those obtained at intervals following x-ray treatment of the scrotum, a procedure shown by Regaud (1906) and Fogg and Cowing (1952) to destroy spermatogonia.

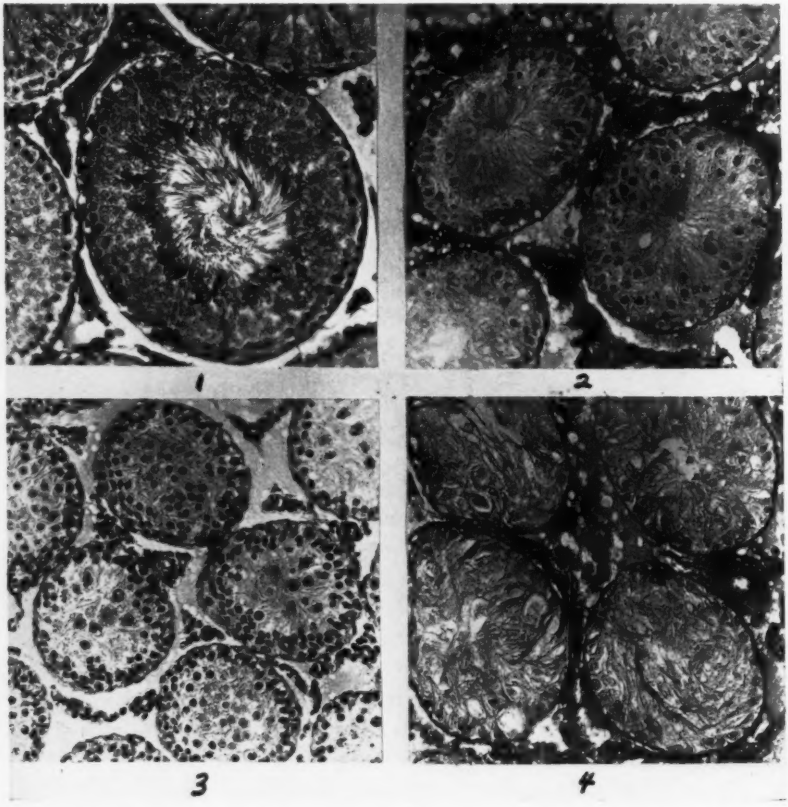

FIG. 2. (1) Normal rat seminiferous tubules. (2) Tubules of rat at end of 30-day Furadroxyl-feeding period. (3) Tubules of rat 42 days after hypophysectomy. (4) Tubules of rat 28 days after x-irradiation of scrotum.

METHODS

Furadroxyl was chosen as representative of the nitrofuran group because it appeared to occupy quantitatively a central position among those studied by the Pauls and their associates. It was fed to the rats at a level of 1.5 gm./kg. of diet.

Oxygen consumption of testicular tissues from rats and men was measured by standard manometric techniques as described by Umbreit *et al.* (1949). Micro Warburg vessels containing approximately 25 mg. (wet weight) of tissue per flask were employed, with pyruvate as substrate. Q_{O_2} was calculated on 15 minute readings during the first hour. There was routinely a lapse of 30 minutes between removal of tissue and the first manometric reading.

The seminiferous tubules from one rat testis were removed and teased apart for us in the microrespirometer vessels. The other testis was placed in Bouin's solution for histologic study.

Hypophysectomized rats were purchased from Hormone Assay Laboratory of Chicago, Illinois. Three mg. testosterone propionate were administered to 6 of these rats for 20 days.

X-irradiation was carried out by exposing the scrota of rats to 600 R for 20 minutes.

Human tubules were taken at biopsy[2] from descended testes and were analyzed at once in the microrespirometers.

RESULTS

Human biopsy material: The results from 12 human subjects are shown in 3 groups in Table 1. In general, the observations of the histologic sections correlated well with the levels of oxidations, both endogenous and with

TABLE 1. OXIDATION OF PYRUVATE IN BIOPSIED HUMAN TESTICULAR TISSUE

Patient number	Q_{O_2}		% stimulation	Spermatogenesis
	Endogenous	With pyruvate		
KL955	0.75	2.50	+233	Normal
KM100	0.60	2.90	+384	Normal
KM134	0.70	1.70	+143	Normal
KM98	2.40	3.00	+ 25	Partial arrest
KM153	1.10	1.40	+ 27	Moderate arrest
KM118	1.90	2.30	+ 21	Normal
KM137	3.60	4.10	+ 14	Normal, but poor motility of sperm
KL958	3.30	3.70	+ 12	Spermatogenic arrest
KM171	2.00	2.20	+ 10	Spermatogenic arrest
KM99	0.20	0.60	+200	Extreme fibrosis
KM147	0.30	0.50	+ 66	Severe fibrosis in 20% tubules, sloughing; impotent
KM154	0.20	1.00	+400	Considerable fibrosis, but good spermatogenesis in surviving tubules

pyruvate as substrate. Normal spermatogenesis was accompanied by the ability of the tubular tissues to oxidize pyruvate well. During spermatogenic arrest, the endogenous level was higher than normal, and the addition

[2] The authors acknowledged the generous cooperation of Dr. R. G. Bunge, Dept. of Urology, in obtaining these biopsies.

of pyruvate did not result in additional oxidations. It is of interest to note that the values for KM118 fit well into the "spermatogenic arrest" group as far as the oxidations are concerned, but this testis was regarded as histologically normal. The last three patients shown are those in which the endogenous oxidations are only half those of the control group. Some stimulation was noted with the addition of pyruvate, but in no case did the absolute values exceed 1.0. In these latter cases considerable fibrosis was present and one subject was described as impotent. Therefore, al-

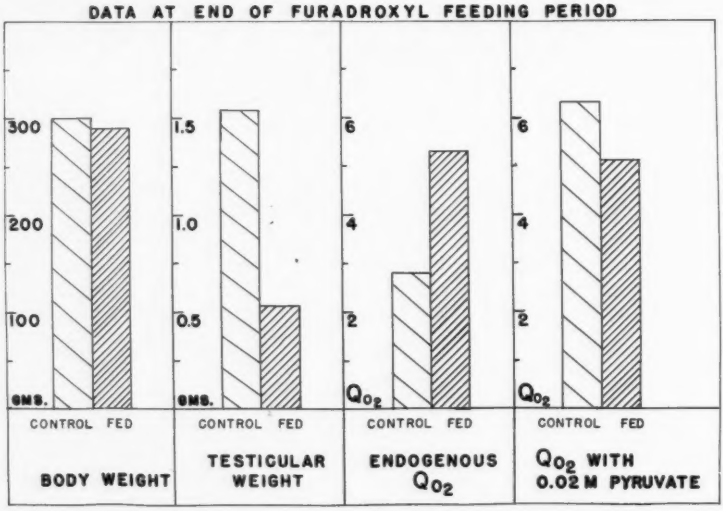

FIG. 3. Data obtained with 16 rats sacrificed at the 60th day of life. Half were fed Furadroxyl in an otherwise normal diet for 30 days prior to sacrifice.

though percentage-wise there was a considerably greater level of oxidations in these tissues when pyruvate was present, the absolute values mirror the existence of abnormalities of spermatogenesis. The data obtained from human patients are consistent, also, with those of Tepperman *et al.* (1949) which showed glucose utilization by normal, but not by the cryptorchid testis.

Rat experiments: In Figure 3 the mean values observed at the end of the 60th day of life, or after 30 days of Furadroxyl feeding, for 8 control and 8 Furadroxyl-fed rats are shown. The histologic differences have previously been cited and are shown in Figure 2, parts (1) and (2). It is apparent from these data that the body weight has not been altered by the Furadroxyl in the diet, but that the testicular weight in the treated animals was only about one-third that of the controls. The endogenous oxygen consumption of the tubules from treated animals was almost twice that the controls. When 0.02 M pyruvate was added, the Q_{O_2} of control tissues more than

doubled the endogenous values, while that of tissues from fed animals was unchanged from its already high endogenous value. Quantitative determinations of pyruvate by the Friedemann-Haugen method (1943) showed that testicular tissues from control but not from fed animals utilized this substrate.

In attempts to explain the high endogenous Q_{O_2} values for tissues from fed animals, glycogen determinations, both chemical and histochemical, were made to detect any residual substrate present. No evidence of increased amounts of glycogen or glucose was found. Also cross-incubation experiments showed little evidence of the presence of a substrate in the poisoned tissue which could be oxidized by normal tissues. Various cofactors known to be necessary for the oxidation of pyruvate, such as coenzymes A, I and II, and ATP, were added singly and together to tissues from fed animals, but failed to restore the ability of these tissues to increase their Q_{O_2}'s in the presence of pyruvate. Apyrase studies showed that the poisoned tissue had no depression of its ability to release the terminal phosphate from ATP.

The recovery period was also studied and some of the data obtained are shown in Figure 4. In the top half of the figure the weight of one testis is plotted against days. The figures by each point indicate the numbers of rats involved in determining the point. It is apparent that the rat testis at 60 days—the beginning of the period shown here—has nearly reached its adult size (curve A). Curve C shows the recovery of testicular weight following Furadroxyl-feeding. The time for half recovery (RT_{50}) was 37 days. The lower plot is that of increased Q_{O_2} with pyruvate, expressed as per cent of endogenous Q_{O_2}. Again curve A does not change during the period of these observations, but curve C shows a gradual return of the ability to oxidize pyruvate, the RT_{50} being about 45 days. These RT_{50} values correspond well with the return of complete spermatogenesis and with the Paul data on glucose utilization.

Attempts were made to hasten recovery by supplying precursors, of necessary cofactors in the form of yeast added to the diet, both during Furadroxyl treatment and/or during the recovery period. This procedure was unsuccessful. Insamuch as the Pauls have shown *in vitro* that succinate can be oxidized in the presence of Furadroxyl, we added 5% succinate to the diet during the Furadroxyl-feeding period for some rats, with the hope that the tubules might have at least part of the energy provided by the Krebs cycle reactions. While the oxidation studies indicated protection in some of the rats, the histologic studies were less encouraging.

To date, the only experimental procedure which has resulted in an increased recovery rate is represented in the two curves labelled "B" in Figure 4. These data were obtained from rats given 100 μg./day estradiol subcutaneously during the Furadroxyl feeding period. Both the testicular

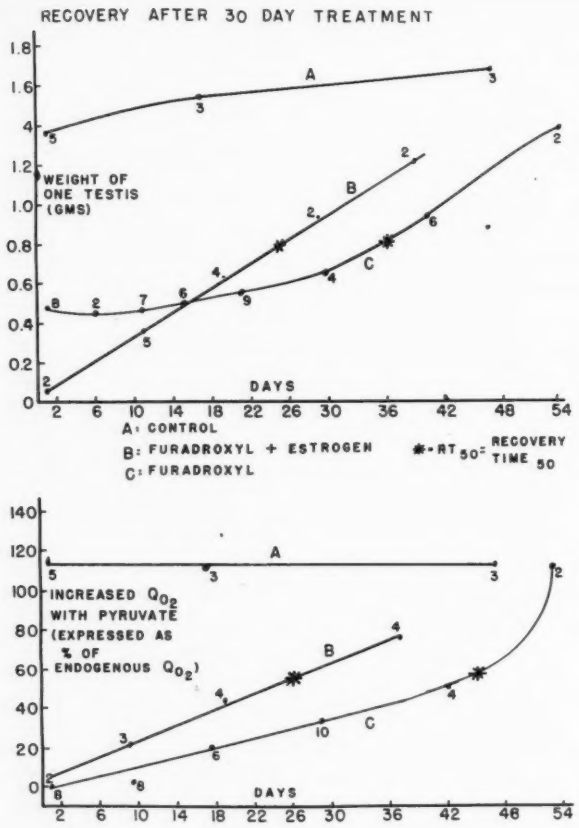

FIG. 4. Data obtained with 3 groups of rats sacrificed at intervals after a 30-day Furadroxyl-feeding period.

size and recovery rate resemble those obtained with estradiol alone, indicating that the action of Furadroxyl may be blocked by the depression of testicular activity with the estrogen. The RT_{50} for both testicular weight and increased Q_{O_2} with pyruvate is approximately 25 days and complete spermatogenesis had returned, as compared to 36–42 days for rats treated with Furadroxyl alone. The effect of estradiol on the testis is well known to be an indirect one through the suppression of gonadotropins and the addition of estradiol directly to the Warburg vessels had no effect, as would be expected.

As a result of these studies and those of the Pauls and others, it appeared that the oxidation of pyruvate in the seminiferous tubules correlated well with the presence of spermatids and mature spermatozoa. When these forms were lacking, the level of endogenous oxidations rose as much as 100%.

In order to test this hypothesis further, two other methods of altering the tubular constituents were chosen for study—hypophysectomy and X-irradiation.

Groups of 4 to 6 rats were sacrificed at weekly intervals following hypophysectomy. The results obtained with 43 rats are shown in the top part of Figure 5, and the typical histologic picture at the end of the study is shown in Figure 2, part (3). A normal range increase in oxidations (+10%) occurred when pyruvate was present until 6 days following hypophysectomy. At 12 days the per cent stimulation had dropped to 33, and continued to drop with time until at 34 days after hypophysectomy there was arrest at the spermatocyte stages and very little increase in oxidations could be induced by adding the pyruvate as substrate in the microrespirometers. The remarks accompanying the data for each period show the gradual depletion of spermatids and mature sperm. After 34–41 days these tubules resembled those of the Furadroxyl-fed rats metabolically, but histologically, secondary as well as primary spermatocytes were present. As in the case of the drug-poisoned tubules, the decreasing differences between endogenous and "pyruvate" values were due in large measure to an elevation of the endogenous values with the loss of the mature sperm forms.

Also shown in this portion of Figure 5 are data for 6 rats treated with testosterone daily following hypophysectomy. After 21–23 days they were sacrificed for tissue study. The testosterone allowed normal maturation of the sperm despite the lack of pituitary hormones, and the oxidation of pyruvate by these tubules was somewhat greater than in control rats.

Another group of 32 rats was treated with x-irradiation in the scrotal area, a procedure known to damage the spermatogonia. The data for these rats are shown in the lower half of Figure 5, and the typical histologic picture at the end of this study is shown in Figure 2, part (4). Groups of rats sacrificed at intervals after the irradiation treatment were shown to have abnormalities progressing with time, but as long as there were considerable numbers of spermatids and mature spermatozoa, the extra oxidation in the presence of pyruvate occurred. After 28 or more days, when these forms were almost completely lacking, the oxidation of pyruvate was similar to that in the tubules of Furadroxyl-fed rats, or in tubules from men suffering from spermatogenic arrest. The histologic differences, however, were that in these irradiated rats the mature forms were not present, nor were the spermatogonia or spermatocytes. The cell types were exclusively Sertoli for the tubules, while the intertubular spaces contained the usual population of Leydig and connective tissue cells. Again the endogenous oxidations were double those of normal tubules.

DISCUSSION

These studies indicate that the oxidation of pyruvate by seminiferous

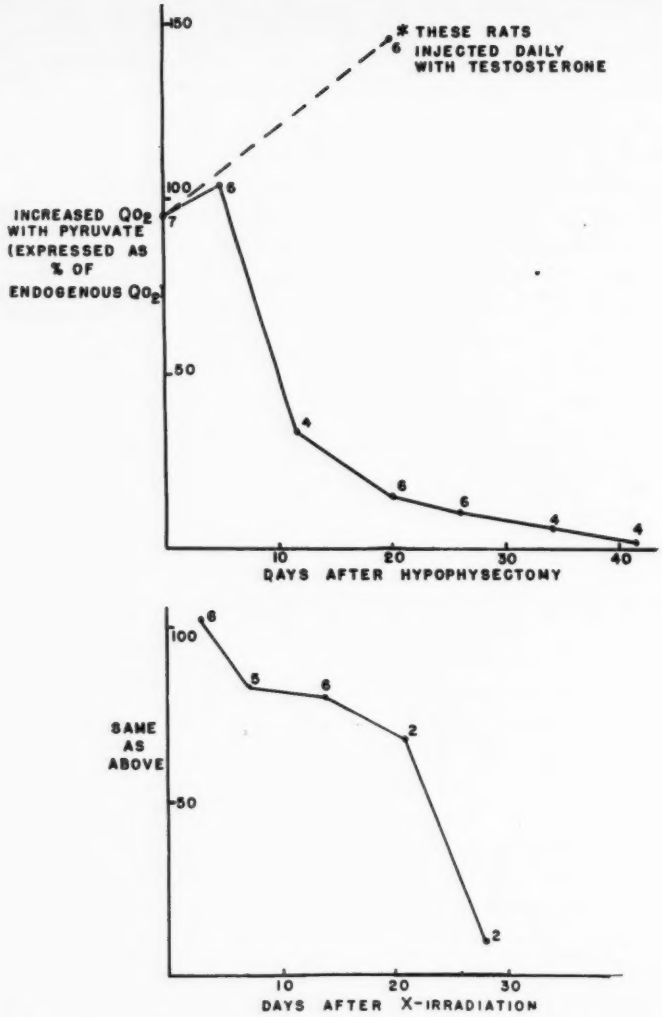

FIG. 5. *Upper graph:* The hypophysectomized rats treated with 3 mg. Testosterone propionate daily depicted a normal spermatogenic process. The 6 day non-testosterone-treated hypophysectomized rats represented histologically normal testes. At 12 days, inhibition of spermiogenesis had taken place and was paralleled by the sharp decline in stimulation of oxidations. Normal and abnormal early phases of spermatids could be found up to 35 days. By 41 days essentially complete arrest at the spermatocyte stages had taken place—see Fig. 2, part (3). *Lower graph:* The testes of the three day X-irradiated rats appeared normal with the exception of the spermatogonia. The 7 day and 14 day post X-irradiation groups show normal sperimiogenesis but spermatogenessis was greatly affected, especially those cells in division which were necrotic. By 21 days the majority of tubules had only spermatids with Sertoli cells. The number of tubules with spermatozoa was greatly reduced. At 28 days after X-irradiation, 60% of the tubules contained only Sertoli cells and the remaining tubules contained only immature spermatids—see Fig. 2, part (4). This depletion of spermatids paralleled the severe drop in stimulation of oxidations.

tubules of rats and possibly of man is carried out primarily by the spermatids and mature spermatozoa.

The nature of the endogenous substrates utilized by the other tubular constituents remains unknown, but it is unlikely that the higher endogenous oxidation levels are due to destruction of glycogen stores.

The histologic and metabolic picture obtained by study of the tubules of the nitrofuran-fed rats may be duplicated by preventing maturation of sperm through hypophysectomy or x-irradiation of the scrotum.

The apparent inhibition of pyruvate oxidations in seminiferous tubules brought about by feeding nitrofurans appears to be merely a reflection of the lack of pyruvate-using spermatids and more mature sperm stages in the tubules. The long recovery period necessary for the return of normal pyruvate oxidation parallels the return of the more mature sperm forms following removal of nitrofuran from the diet.

These studies suggest that the primary nitrofuran effect in the seminiferous tubules does not lie among the catabolic reactions of the "energy wheel", but may be found by further study in the area of the synthetic reactions involved in the maturation of cells beyond the primary spermatocyte stage. It is possible that consequent to the metabolic defect in the processes leading to sperm maturation, the primary spermatocytes are unable to secure the requisite energy to complete their meiotic division and as a result remain in a state of arrest.

It is hoped that further studies in this field will be fruitful in understanding and solving the problems of male infertility.

SUMMARY

The chronic feeding of nitrofurans to rats in an otherwise normal diet resulted in a loss of ability of the seminiferous tubules to oxidize pyruvate *in vitro*. The development of this state paralleled the development of spermatogenic arrest at the primary spermatocyte stage. Recovery of ability of the tubules to oxidize pyruvate following withdrawal of the nitrofuran also paralleled the return of spermatids and mature spermatozoa to the tubules. The coupling of the oxidation of pyruvate to the presence of mature sperm forms was duplicated in rats after hypophysectomy or after x-irradiation of the scrotum. Therefore, it seems probable that the apparent inhibition of substrate oxidations in seminiferous tubules by nitrofurans is not the primary action of these compounds and that this primary action must be sought in the reactions involved in the maturation of primary spermatocytes. Parallelisms between Furadroxyl-fed rats and men with spermatogenic arrest with regard to pyruvate oxidation of their tubules is apparently due merely to the lack of the more mature cell forms in both.

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THE SPECIFICITY OF THYROXINE BINDING BY SERUM ALPHA GLOBULIN[1]

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CIRCULATING thyroxine associates with a specific alpha globulin which has an electrophoretic mobility at pH 8.6 intermediate between alpha-2 and alpha-1 (Larson, Deiss, and Albright, 1952; Winzler and Notrica, 1952; Robbins and Rall, 1952; Horst and Rosler, 1953; Larson, Deiss, and Albright, 1954). This selective binding can be demonstrated in vitro by adding I^{131}-labelled thyroxine to serum provided the maximal binding capacity of the protein is not exceeded (Robbins, Rall, and Petermann, 1954, Albright, Larson, and Deiss, 1954). Triiodothyronine is also bound by this protein but there is evidence that this bond is somewhat weaker (Deiss, Albright, and Larson, 1953). This observation led to the examination of other analogues of thyroxine in an effort to determine the specificity of thyroxine binding by serum alpha globulin.

MATERIAL AND METHODS

The technic employed was the measurement of the extent of removal of a quantity of labelled L-thyroxine from its binding site by equal and excess quantities of the analogues. Comparisons were then made with the removal by non-labelled DL-thyroxine. A quantity of labelled L-thyroxine was selected (in this case 0.1 μg.) which would be distributed equally between the thyroxine binding globulin and albumin sites.

The serum used in this study was obtained from a normal subject. A single preparation of chromatographically pure I^{131}-labelled L-thyroxine[2] was used throughout. The analogues examined are given in Table 1.

Preparation of Solutions of Analogues

DL-thyroxine and the analogues, with the exception of thyroxamine, were dissolved in 0.1N NaOH containing 20% propylene glycol. Thyroxamine proved insoluble in this solvent, but dissolved in a mixture of 50% propylene glycol and 0.2N NaOH. Dilutions were prepared, using 0.1N NaOH containing 20% propylene glycol, to yield solutions containing equal, 10 and 100 times the molar equivalents of 1 μg. of thyroxine per ml.

Preparation of Analogue-Serum Mixtures for Electrophoresis

0.1 ml. of each of the analogue solutions and 0.01 ml. of a solution containing 0.1 μg of radiothyroxine were added to 1 ml. of serum. The mixture was allowed to stand for 20 minutes at room temperature with frequent agitation. In a control experiment 0.1

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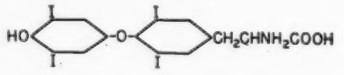
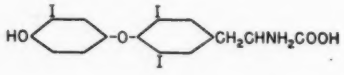
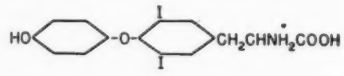
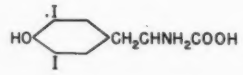
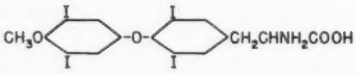
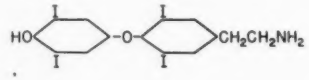
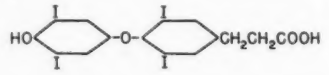
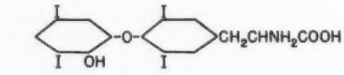
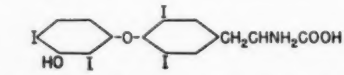
[1] This work was supported in part by the Wisconsin Alumni Research Foundation.

[2] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

TABLE 1. STRUCTURE OF THYROXINE ANALOGUES

Thyroxine[1]	
Triiodothyronine[2]	
Diiodothyronine[3]	
Diiodotyrosine	
Methoxythyroxine[4]	
Thyroxamine[4]	
Propionic Acid Analogue[4]	
"Ortho" thyroxine[4]	
"Meta" thyroxine[4]	

Compounds were furnished through the kindness of: 1. E. R. Squibb and Sons. 2. Dr. M. L. Schiffrin, Hoffmann-LaRoche Company. 3. Dr. Arthur Heming, Smith, Kline and French Laboratories. 4. Dr. Henry Lardy, Enzyme Institute, University of Wisconsin.

ml. of a 20% propylene glycol-0.1N NaOH solution was used. 0.05 ml. of each of these mixtures was subjected to paper zone electrophoresis using 0.1 M veronal buffer pH 8.6 previously described (Deiss, Albright and Larson, 1952).

Counting Technic

At the completion of electrophoresis the strip was dried and radioactivity determined by use of a chromatogram scanner employing an Esterline Angus recorder. The distribution of radio-activity was measured by computing with a planimeter the area under the curves produced by the recorder.[3] After counting the protein bands were identified by staining the strip by the bromphenol blue elution method.

RESULTS

The data are presented in Table 2. The values are expressed as the percentage of labelled L-thyroxine remaining at the thyroxine binding

[3] The relationship of the area under the curves to the amount of radioactivity on the paper has been shown to be directly proportional.

TABLE 2. PER CENT OF RADIOACTIVE L-THYROXINE REMAINING IN THYROXINE BINDING GLOBULIN AFTER ADDITION OF EQUIMOLAR, AND 10 AND 100 TIMES MOLAR QUANTITIES OF THE INDICATED ANALOGUE*

Analogue	Molar equivalents			
	0	1	10	100
DL-thyroxine[1]	52	47	19	3
D-thyroxine[4]	—	47	36	13
L-triiodothyronine[2]	—	48	33	12
L-diiodothyronine[3]	—	48	47	47
diiodotyrosine	—	50	45	45
methoxythyroxine[4]	—	46	44	28
thyroxamine[4]	—	50	44	25
propionic acid analogue[4]	—	50	46	43
"ortho"-thyroxine[4]	—	50	44	31
"meta"-thyroxine[4]	—	50	48	39
iodide	—	52	50	52

Compounds were furnished through the kindness of: 1. E. R. Squibb and Sons. 2. Dr. M. L. Schiffrin, Hoffmann-LaRoche Company. 3. Dr. Arthur Heming, Smith, Kline and French Laboratories. 4. Dr. Henry Lardy, Enzyme Institute, University of Wisconsin.

* The 20% propylene glycol—0.1 *N* NaOH solution used in dissolving the analogues produced no displacement of L-thyroxine from the binding globulin.

globulin zone after the addition of equimolar, and 10 and 100 times the molar quantities of the indicated analogue.

DL-thyroxine added in equimolar concentration had very little effect on the distribution of the radioactivity. When 10 times the amount of non-radioactive DL-thyroxine was added, only 19% of the labelled L-thryoxine remained at the alpha zone. The addition of 100 times the quantity of non-radioactive DL-thyroxine removed all but 3% of the labelled L-thyroxine from this zone.

Among the analogues examined triiodothyronine and D-thyroxine[4] had the greatest effect on the distribution of the radioactivity. After the addition of 100 times the molar quantities of these analogues, 12% and 13% respectively of the labelled L-thyroxine remained at the thyroxin binding globulin zone. The other analogues at 100 times the molar concentration had less effect on the distribution of the radioactivity. They may be arranged in order of decreasing effectiveness as follows: thyroxamine (25%), methoxythyroxine (28%), "ortho"-thyroxine (31%) "meta"-thyroxine (39%) and propionic acid derivative (43%). Diiodotyrosine, diiodothyronine and iodide had no significant effect.

DISCUSSION

The removal of the labelled thyroxine from the binding globulin following the addition of excess unlabelled thyroxine (or analogues) occurs regardless of the sequence of addition. This is assumed to indicate not only that the binding globulin has a limited capacity to bind thyroxine as reported previously (Albright, Larson and Deiss, 1955), but that an

[4] Kindly supplied by Dr. Henry Lardy, Enzyme Institute, University of Wisconsin.

equilibrium exists between the thyroxine bound to the thyroxine binding globulin, that bound to albumin, and probably a very small quantity in free solution.

Removal of the labelled thyroxine from the globulin zone by thyroxine analogues is taken as evidence of competition for the binding sites on this specific protein. The displacement of the radiothyroxine by unlabelled thyroxine provides a standard for comparison of the relative binding affinity of the analogues.

The three groups, amino, carboxyl and phenolic, appear to be essential for the binding of thyroxine since the absence of any one of them reduces markedly the binding affinity of the analogue. The amino group appears to contribute the most since the deaminated analogue binds very poorly, while the decarboxylated analogue, thyroxamine, retains a slight binding affinity.

The decreasing affinity of thyroxine, triiodothyronine and diiodothyronine for the binding globulin probably reflects the influence of the iodine atoms at the 3′ and 5′ positions on the dissociation of the phenolic groups. The dissociation constant of the phenolic group for these three compounds has been found to be: thyroxine 6.5, triiodothyronine 9.5 and diiodothyronine 10.0 (Lardy, 1954).

The absence of binding of diiodotyrosine and the reduced affinity of the isomers, "meta-" and "ortho-" thyroxine and the stereoisomer D-thyroxine, provides evidence that there is a critical spatial distribution of the binding sites on the protein. The failure to bind D-thyroxine strongly may also explain the observed differences in volume distribution between the stereoisomers of thyroxine (Rall, Robbins, Becker and Rawson, 1953).

The nature of the binding sites on the protein itself is obscure. The presence of sulfhydryl groups has been excluded, however, as it has been observed that treatment of serum with mercuro-chloro-benzoate does not affect the binding affinity for thyroxine (Larson and Albright, 1954).

SUMMARY AND CONCLUSIONS

The specificity of the binding of thyroxine by thyroxine binding serum globulin was examined. The technic employed was the measurement of the extent of displacement of a small quantity of labelled *l*-thyroxine from this protein by equal and excess quantities of thyroxine analogues.

All of the analogues were found to have less affinity than thyroxine for this protein. Among the analogues triiodothyronine and D-thyroxine showed the greatest affinity; the others in decreasing order of affinity were thyroxamine, methoxythyroxine, "ortho"-thyroxine, "meta"-thyroxine, and the propionic acid analogue. Diiodotyrosine, diiodothyronine and iodide had no significant binding effect.

The amino, carboxyl and phenolic groups are essential for the binding of thyroxine, with the amino group being the most significant. The influence

of iodine atoms at the 3′ and 5′ positions on the dissociation of the phenolic group is consistent with the decreasing affinity of thyroxine, triiodothyronine and diiodothyronine for binding globulin.

A critical spatial distribution of the binding sites on the serum protein is suggested by the absence of binding by diiodotyrosine and reduced binding of the "meta"- and "ortho"-thyroxine, and D-thyroxine isomers.

The binding of thyroxine binding globulin is highly specific.

Acknowledgment

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THE SEVENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM SPONSORED JOINTLY BY THE ENDOCRINE SOCIETY AND THE INDIANA UNIVERSITY SCHOOL OF MEDICINE AT THE CONTINUATION STUDY CENTER OF THE INDIANA UNIVERSITY MEDICAL CENTER, INDIANAPOLIS, INDIANA— SEPTEMBER 26–OCTOBER 1, 1955

The faculty will consist of twenty-one eminent clinicians and investigators from various parts of the country in the fields of endocrinology and metabolism. The program will cover the various endocrinopathies with emphasis on the clinical aspects, with demonstrations of laboratory tests, presentations of cases, and question and answer panel discussions. The course is designed to cover the main aspects of diagnosis and therapy in the field of endocrinology and metabolism for the physician in general practice and for those in other specialties who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of lectures will be available to the registrants at the time of the assembly.

For further information concerning the program, write Post Graduate Office, Indiana University Medical Center, 1100 West Michigan Street, Indianapolis 7, Indiana. Registration is limited to 100; tuition fee is $100.00. Arrangements for housing accommodations will be made through the Indiana Medical Center.

GORDON RESEARCH CONFERENCE, AAAS

AUGUST 1–5, 1955

KIMBALL UNION ACADEMY, MERIDEN, NEW HAMPSHIRE

THE STRUCTURE, CHEMISTRY, AND PHYSIOLOGY OF BONES AND TEETH

W. F. NEUMAN, *Chairman*
R. F. SOGNNAES, *Vice Chairman*

Individuals interested in attending the Conference are requested to send in their applications to the Director. Each applicant must state the institution or company with which he is connected and the type of work in which he is most interested. Attendance at each Conference is limited to 100.

Requests for attendance at the Conferences, or for any additional information should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From June 10 to September 2, 1955, mail should be addressed to Colby Junior College, New London, New Hampshire.

ENZYME SYMPOSIUM

A 3-day International Symposium on ENZYMES: UNITS OF BIOLOGICAL STRUCTURE AND FUNCTION, sponsored by the Henry Ford Hospital and The Edsel B. Ford Institute for Medical Research will be held in the auditorium of Henry Ford Hospital, November 1, 2, and 3, 1955.

Interrelationships between enzymology and other fields, notably genetics, physiology, biochemistry, and pharmacology, will constitute the general theme of the Symposium. The specific topics for the six sessions will be: Origin of Enzymes, Status of the Gene-Enzyme Relationship, Enzymes and Cell Structure, Enzymatic Basis of Some Physiological Functions, Cellular Energy Sources, and Regulation of Enzyme Activity. More than thirty internationally known scientists have accepted invitations to participate.

Interested persons may secure a copy of the Preliminary Announcement by writing to Dr. Clarence E. Rupe, Henry Ford Hospital, Detroit 2, Michigan.

Invitations will be sent to as many as can be accommodated.

BOOK REVIEW

THE HYPOPHYSEAL GROWTH HORMONE, NATURE AND ACTIONS. International Symposium, 1955. Editors, R. W. Smith, Jr., O. H. Gaebler and C. N. H. Long. Published by the Blakiston Division of McGraw-Hill Book Company, Inc., New York.

This volume comprises the proceedings of a stimulating symposium devoted to the hypophyseal growth hormone held in Detroit in October, 1954 under the auspices of the Henry Ford Hospital and the Edsel B. Ford Institute for Medical Research. Space precludes a listing of the thirty major contributions to the symposium and their respective authors. Suffice it to say that all current areas of investigation in the field are discussed comprehensively as well as intensively in terms of bioassay, preparation, and physiochemical properties of growth hormone on the metabolism of proteins, fats and carbohydrates. In addition, the effects of growth hormone on various organs and cellular systems are expertly covered in the light of present knowledge.

This volume, while emphasizing many of the current uncertainties pertaining to the physiological and metabolic effects of growth hormone, should be of unusual usefulness to workers in the field by virtue of the breadth and detail of its coverage. The absence of an index is regrettable but does not detract importantly from the value of the book.

ERNEST KNOBIL

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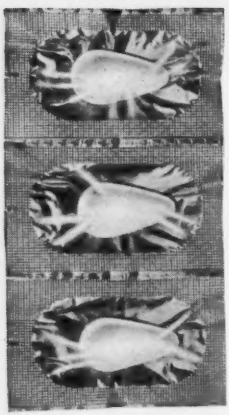

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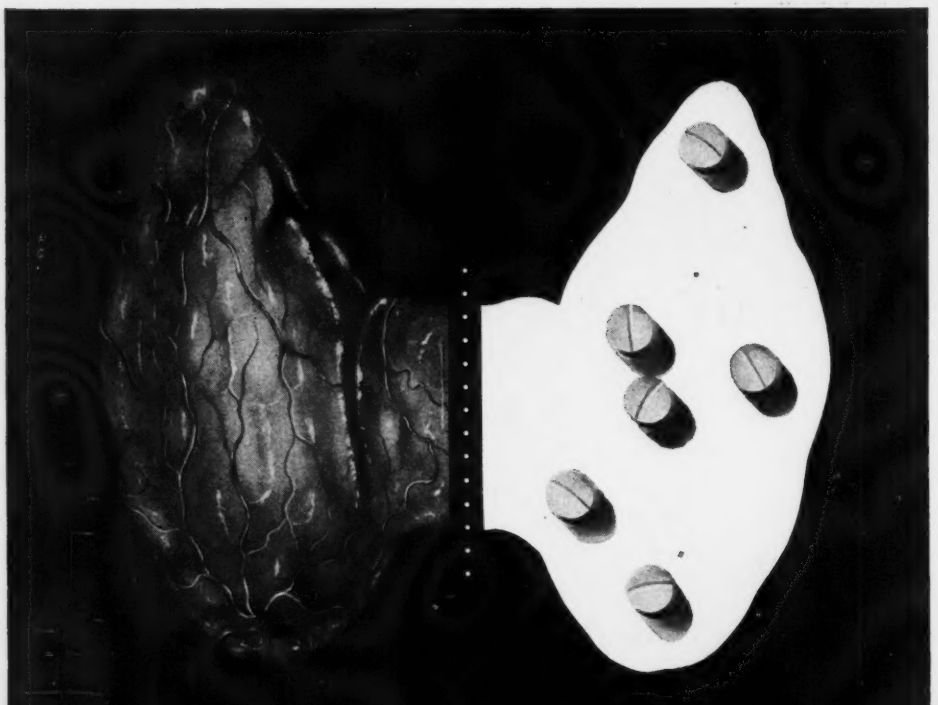

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